Water stress and disease development in *Eucalyptus marginata* (jarrah) infected with *Phytophthora cinnamomi*.

This thesis is presented for the degree of Doctor of Philosophy in the Department of Biological Sciences, Murdoch University, Perth, Australia

by

Anne Lucas BSc (Hons)

2003

Frontispiece: Botanical illustration of *Eucalyptus marginata* (jarrah).

Originally published in "Eucalyptographia: a descriptive atlas of the eucalypts of Australia and the adjoining islands" by Ferdinand von Mueller: Government Printer, Melbourne, 1880. Reproduced with permission from the Library of the Royal Botanic Gardens, Melbourne.

Declaration

I declare that this thesis is my own account of my research and contains, as its main content, work which has not previously been submitted for a degree at any tertiary institution.

Anne Lucas
March, 2003

Abstract

The south-west of Western Australia has a Mediterranean climate and flora endemic to this area, including the keystone species, jarrah (*Eucalyptus marginata*), have adapted to the droughted summer conditions. The introduction of an exotic soilborne pathogen, *Phytophthora cinnamomi*, has challenged the survival of this and many other species. The expectation might be that plants stressed by drought are more susceptible to disease and this study examined the development of disease caused by *P. cinnamomi* in *E. marginata* and the significance of water status to that development. Seedlings of *E. marginata*, clonal plants resistant to *P. cinnamomi* and clonal plants susceptible to *P. cinnamomi*, were subjected to different watering regimes in a number of field and glasshouse experiments. To determine the level of drought stress that could be imposed on container-grown *E. marginata* seedlings without killing them, a preliminary experiment progressively lowered the moisture levels of the substrate in their containers, until the plants reached wilting point, at which time moisture was restored to a predetermined droughted level and the process repeated. With each subsequent droughting the wilting point was lower until it was found that the seedlings could survive when only 5% of the moisture lost from container capacity to wilting point was restored. No deaths had occurred after seedlings had been maintained at this low level for 14 days (Chapter 2). Based on these findings, the level of droughting maintained in all experiments conducted under controlled glasshouse conditions was 10% restoration.

After testing the appropriateness of underbark inoculation, and a zoospore inoculation method for which no wounding was necessary, a new, non-invasive stem inoculation technique was developed. Stems were moistened in a pre-treatment, then agar plugs colonized with *P. cinnamomi* mycelium were held against the stem with wads of wet cotton wool and bound in place with tape. This technique resulted in a high proportion of infection in *E. marginata* (Chapter 4) without the need for underbark inoculation or the use of zoospores (Chapter 3). It was successfully used in a large field trial in a rehabilitated bauxite mine site with 2-year-old *E. marginata* clonal plants, resistant to *P. cinnamomi* (Chapter 5). Inoculation was in late spring after the winter and spring rainfall. This timing was to allow comparison of disease development in stressed plants under normal droughted summer conditions compared with its

development in non-stressed, irrigated plants. However, two months after inoculation, the area was deluged with unseasonal and abnormally heavy summer rainfall, negating any difference in the treatments and causing an outbreak of *P. cinnamomi* in the soil from an adjacent infested site. This resulted in the infection and death of some non-inoculated control clones. Monitoring of the site continued for twelve months and the advance of *P. cinnamomi* at the site was mapped.

To test the effect of drought on the expression of *P. cinnamomi* under more controlled conditions, a series of glasshouse experiments was set up that simulated two possible summer conditions; drought or drought followed by abnormally high summer rainfall. These experiments utilised *E. marginata* seedlings and clonal plants, some resistant and some susceptible to *P. cinnamomi*. Plants were inoculated with *P. cinnamomi* prior to or after droughting. Results were compared to those of control plants that had not experienced water deficit. In both seedlings and clonal plants, the greatest extent of colonization was found in plants which had experienced no water deficit. These results indicated that drought stress played a role in inhibiting the *in planta* development *of P. cinnamomi* in all genotypes (Chapter 8). This finding was consistent for both clones, susceptible and resistant to *P. cinnamomi*. Most recoveries were made from non-stressed clonal plants, resistant to *P. cinnamomi* (Chapter 6) and more colonization was found in non-stressed clonal plants, susceptible to *P. cinnamomi* (Chapter 7), than was recorded for droughted plants.

The results of the field trial showed that *P. cinnamomi* was not recovered from some inoculated stems, which had obvious lesions, when segments were plated onto selective agar. This led to an intensive *in vitro* investigation into improved methods of recovery. Dark brown exudates from some segments of inoculated stems stained the surrounding agar onto which they were plated, suggesting the presence of phenolic compounds. Recovery of the pathogen from stems increased by about 10% when segments were first soaked in distilled water to leach out the phenolic compounds, then replated onto agar. Other recovery methods were also tested, including (1) baiting with *Pimelea ferruginea* leaves floated on the surface of water or soil filtrate, in which the infected stem segments were immersed and (2) the application of different light and temperature regimes. It was clearly shown that exudates from infected stems of field grown *E. marginata* inhibited the outgrowth of *P. cinnamomi* onto the agar. To counter the possible toxic effect that oxidized phenolics had on the growth of the *P. cinnamomi*, an antioxidant was added to the agar. *P. cinnamomi* was grown on media which

incorporated exudates from infected stems and different concentrations of ascorbic acid, with and without adjusted pH levels. There was a pronounced pH effect, with less growth on media with lower pH and no significant increase in growth of the mycelium with increased ascorbic acid concentration on pH adjusted agar (Chapter 9).

The inhibitory effect of the exudates from the stem segments led to an investigation of the possibility that, if seedlings to be planted in the rehabilitation process could be pre-treated with phenolic compounds to render them more resistant, they may have an advantage when establishing in areas where there was a potential threat of *P. cinnamomi*. *E. marginata* seeds were germinated and the seedlings grown hydroponically in a constant temperature growth room. Different concentrations of synthetic catechol, a phenolic compound naturally occurring in *E. marginata*, were added to the nutrient solution. Roots remained immersed in the catechol solutions for three days, before being inoculated at the root tip with zoospores of *P. cinnamomi*. Roots in higher concentrations of catechol were less colonized than those in lower concentrations, indicating an increased resistance to the pathogen (Chapter 10). Further work is required to determine if seedlings treated before being planted in areas threatened by an outbreak of *P. cinnamomi* have a greater capacity for survival, and for how long the protection persists. The improved recovery of *P. cinnamomi* from infected plants is important for accurate assessment of the spread of the disease in an area and for the subsequent implementation of management strategies of containment and control. An outbreak of *P. cinnamomi* can impact on the revegetation of rehabilitated mine sites and the aetiology of the pathogen in mine sites needs to be more fully understood.

The interaction of plant defences with the invasive pathogen has been examined in a range of environments in the field, the glasshouse, in a hydroponics system and *in vitro*. The results indicate that summer droughting increases the resistance of *E. marginata* to *P. cinnamomi*. However, more work is required to understand the mechanisms involved. The study also indicates that clones of *E. marginata*, selected as resistant to *P. cinnamomi*, are not resistant under all conditions and that environmental interactions should be further investigated. Lastly, for effective management strategies to be implemented it is critical that the pathogen can be confidently isolated from plants. It was shown that exudates from infected hosts inhibit the recovery of *P. cinnamomi*. Recovery methods that can overcome these inhibitory compounds are required. The findings invite further research into the complexity of host-pathogen relationships.

List of Publications and Presentations

Lucas, A. McComb J., Colquhoun, IJ and Hardy G.E.StJ. (2002) A new non-invasive technique for inoculating plants with *Phytophthora cinnamomi*. *Australasian Plant Pathology* 31 (1) 27-30.

Lucas, A. McComb J., Colquhoun, IJ and Hardy G.E.StJ. (2001) Summer rainfall and the development of disease caused by *Phytophthora cinnamomi* in droughted *Eucalyptus marginata* plants. Poster presentation at Second International IUFRO Meeting, Albany, Western Australia, 2001. (and Abstract)

Lucas A. (2001) Our Conference Logo – The Splendid Blue Fairy-wren*, Malurus splendens splendens*, Maluridae (Quay and Gaimard 1830). Proceedings of the Second International IUFRO Meeting, Albany, Western Australia, 2001.

Table of Contents

Abbreviations

ALCOA	Alcoa World Alumina (Australia)
ANOVA	Analysis of variance
CALM	Department of Conservation and Land Management (WA).
CC	Container capacity
Ctrl	Control
DT	Diseased tissue
DW	Dry weight
FW	Fresh Weight
HT	Healthy tissue
Inoc	Inoculation or inoculated plant
ROI	Region of inoculation
RWC	Relative water content
SDW	Sterile distilled water
SEM	Scanning electron microscopy
SS	Clonal plants, susceptible to *P. cinnamomi*. The SS clonal line used in Chapters 7 and 8 of this thesis was *E. marginata* 11J402.
RR	Clonal plants, resistant to *P. cinnamomi*. The RR clonal lines used in this thesis were *E. marginata* 77C40 (Chapter 5) and 1J30 (Chapters 6 and 8).
S	Seedlings of *E. marginata* (Chapters 2, 3, 4 and 8).
TW	Turgid weight
WP	Wilting point
XPP	Xylem pressure potential

Colour Key:

As far as possible, consistent representation of inoculated plants and the corresponding control plants in each of the three watering regimes are presented in the results throughout the thesis as shown below:

Watering regime 1 Inoculated seedlings kept at container capacity (CC) throughout the trial.

Watering regime 1 Control (sham-inoculated) seedlings kept at container capacity (CC).

Watering regime 2 Inoculated seedlings droughted to wilting point (WP) then maintained until harvest at a droughted level, with 10% of substrate moisture lost from CC to WP restored.

Watering regime 2 Control (sham-inoculated) seedlings droughted as in Watering regime 2.

Watering regime 3 Inoculated seedlings droughted as in 2, but restored to CC after 14 days of droughted conditions.

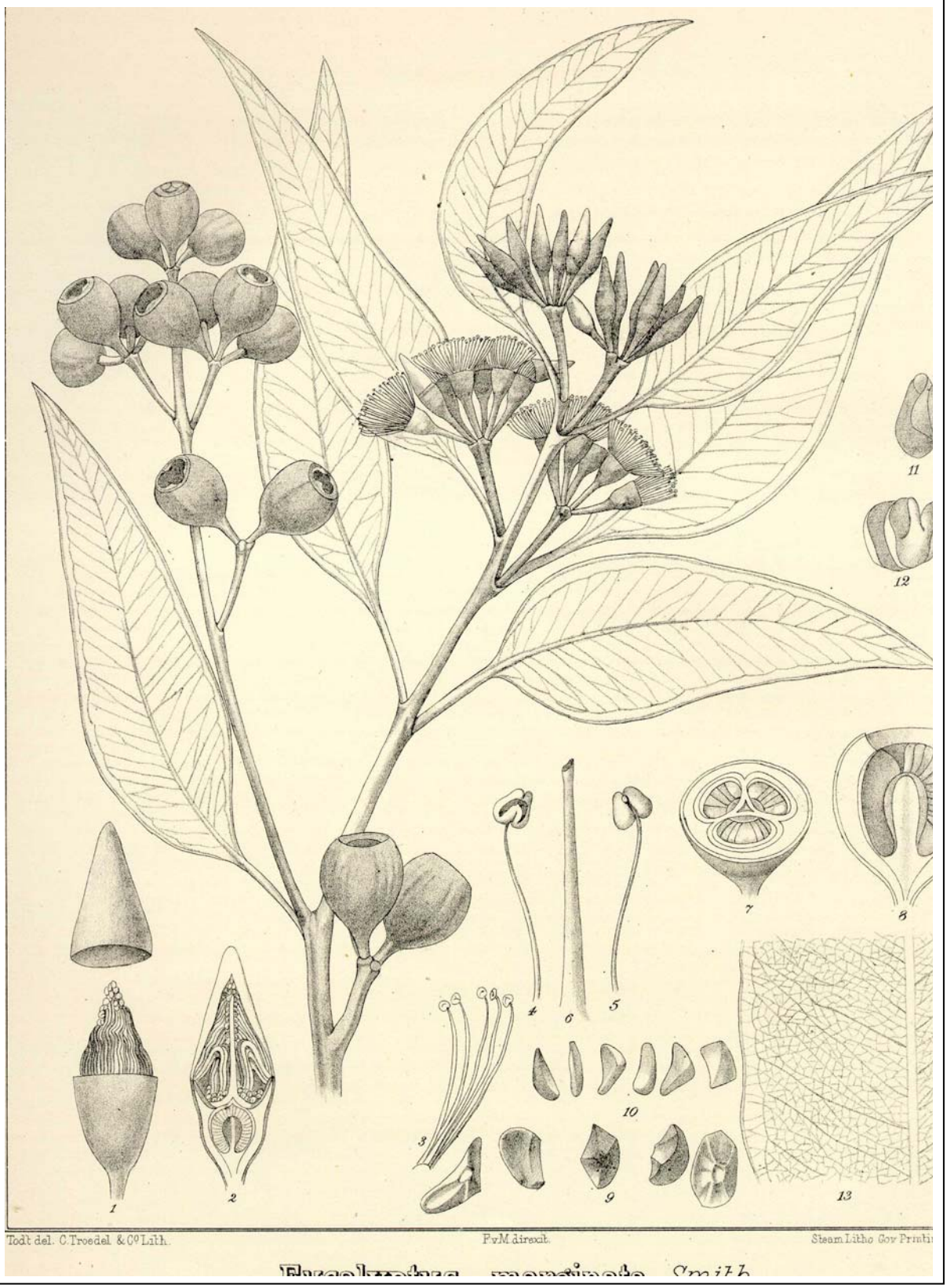
Watering regime 3 Control (sham-inoculated) seedlings droughted then restored to CC as in Watering regime 3.

Acknowledgements

Sincere thanks go to my supervisors, Dr Giles Hardy and Professor Jen McComb of Murdoch University and Dr Ian Colquhoun of Alcoa World Alumina of Australia. They have provided guidance and support throughout the project.

I would like to thank Alcoa World Alumina for the contribution of financial and in kind support which enabled the research to be completed. I would also like to thank Murdoch University for the supplementary scholarship, which allowed me to complete the writing of the thesis. Numerous people have contributed to the outcome and to all those who assisted in the laboratory, the glasshouse and especially in the field in all weathers, I would like to express my appreciation. Special thanks must go to Associate Professor Mike Calver for advice with statistical analyses and to Gordon Thompson for training and assistance with histology and photography. I am also grateful for the helpful advice from colleagues, technicians, staff in the office, library and at the mine site and from tutors in the post-graduate programs.

Special acknowledgement of the unconditional support given by my family and some close friends is due. I especially acknowledge the support of the late Professor Mike Cullen, whose friendship will always be an inspiration to me. His interest and encouragement prompted me to apply for this research project and his constant reassurance and unfailing logic were invaluable.


11
12
7
8
4
6
5
10
3
9
13
1
2
Todt del. C.Troedel & Co Lith.
P.v.M. direxit.
Steam Litho Gov Printi

Chapter 1
General Introduction and Literature Review

The jarrah forest evokes a broad spectrum of response from different sectors of the public. A biologically diverse ecosystem, with a high degree of floral and faunal endemism, it has become the subject of economic, political, scientific and social interests. The growing awareness of the presence of an exotic pathogen and the severity of its impact on forest species, has concerned all sectors, prompting increased interest. Located in the south-west of Western Australia, the jarrah forest community has evolved while having to contend with edaphic and environmental factors, including nutrient-poor soil, fire and drought. The focus of this current study is the interaction between an endemic, dominant canopy species, jarrah (*Eucalyptus marginata*), and an exotic pathogen, *Phytophthora cinnamomi*, under different watering regimes.

1.1 The jarrah forest

Location

Confined to a narrow geographic range (longitude 115°50' to 116°50' E), the jarrah forest has a varied topography. Most of its western margin closely follows the Darling Scarp, an escarpment running roughly parallel with the coastline of Western Australia, and it extends from north east of Perth to Walpole in the south of the state (latitude 31° 51' to 33°30' S) (Fig. 1.1).

Geology and soil

The Yilgarn Craton is an uplifted plateau, where the Darling Range batholith rises 250 to 400 metres above sea level with granite outcrops and monadnocks rising another 150 metres above the plateau (Bell and Heddle, 1989; Nemchin and Pidgeon, 1999). The craton has tilted with greater uplift in the west forming the Darling Scarp and exposing granites overlain with laterite (Mulcahy *et al*., 1972; Nemchin and Pidgeon, 1999). Deep V-shaped valleys occur near the western margins with broader valleys in the eastern area of the forest (Shearer and Tippett, 1989).

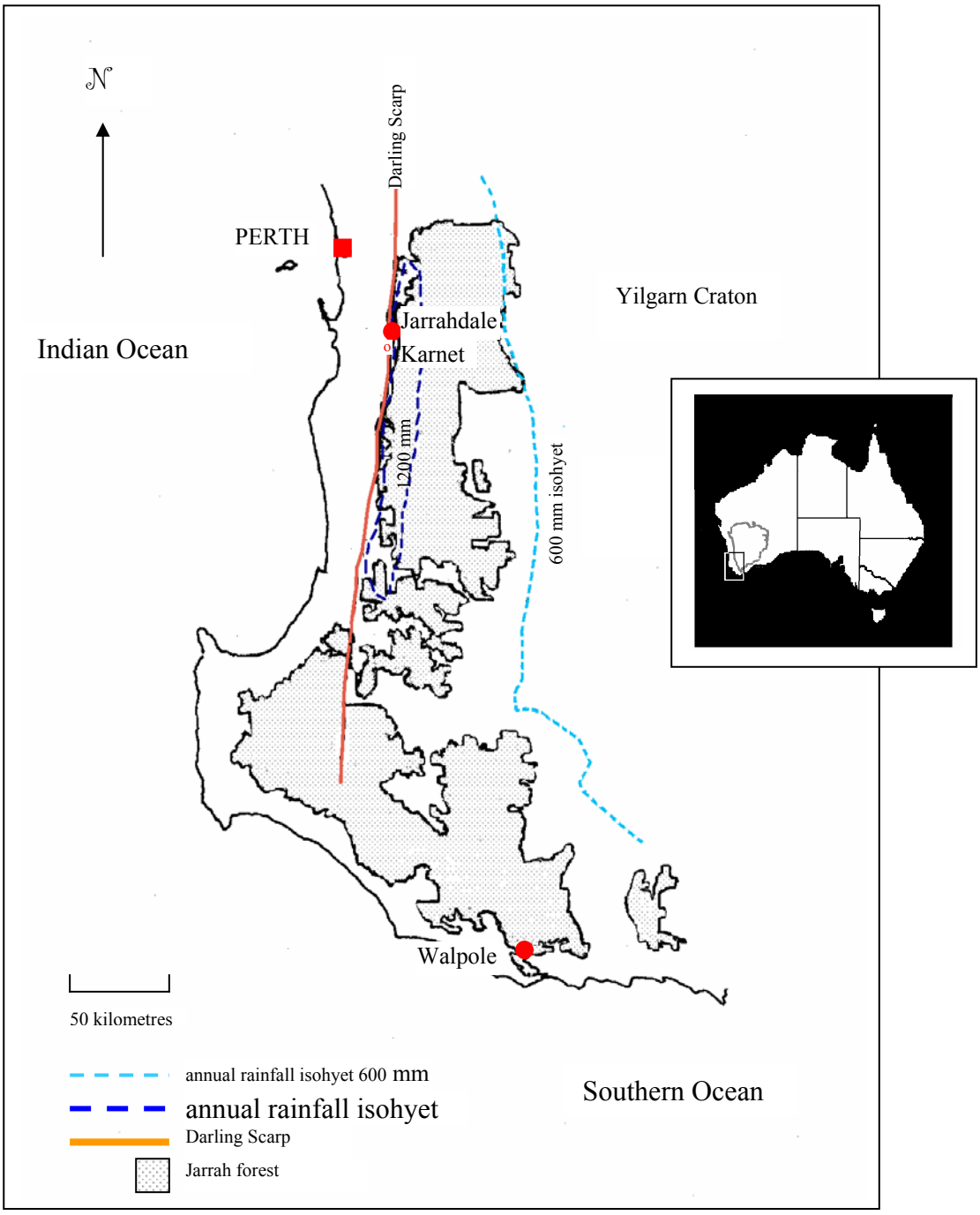


Figure 1.1 Sketch map of the south-west of Western Australia, showing the location of the jarrah (*Eucalyptus marginata*) forest in relation to the Darling Scarp (western edge of Yilgarn Craton) and to annual rainfall. Field work was conducted at the Jarrahdale mine site of Alcoa World Alumina (Australia) and daily weather records were supplied by the Bureau of Meteorology from their station at Karnet.

Inset: Map of Australia showing extent of the Yilgarn Craton in Western Australia (W.A.) and the locality of the main map.

The soil profile of the uplifted plateau has features which strongly influence the hydrology of sites in the jarrah forest and has implications for the current study (Chapter 5). Typically, below the sandy, gravelly soils of the A horizon, the B horizon is a layer of laterite, about 4 to 5 metres thick. This weathered, residual, igneous rock is composed of oxides of aluminium and iron and can form a hard duricrust. Below this, kaolinitic clays extend to the bedrock (Nichols *et al*., 1985; Kinal *et al*., 1993). Where deposits of bauxite (aluminium oxide) are friable and the alumina content is high, the area has been targeted for mining. This operation removes a layer about four metres deep (Ward *et al*., 1996). Clearing of land results in changes in the composition, nutrients and salinity of the soil; reduced evapotranspiration and redesigned topography alter the hydrology (Armstrong, 1979; Colquhoun and Hardy, 2000) which is determined not only by the high infiltration capacity of the gravelly soils but also by the impediment to subsurface flow by the concreted lateritic duricrust and the impermeable claypans (Kinal *et al*., 1993). Fissures in the laterite can facilitate the penetration by tree roots (Doley, 1967;) and allow limited percolation of water to horizons deeper in the profile (Dell *et al*., 1983; Kinal *et al*., 1993). Generally low in essential nutrients of N, P and K, the leached soils have a pH of 6.5 to 5.0 (Podger, 1968). On a rehabilitated bauxite mine site, pH of soils ranged from 5.3 to 5.7 (Ward and Koch, 1996).

Climate

A mediterranean climate prevails in the region. Normally, seasons are distinct with cool wet winters preceding long, hot, dry summers. Annual rainfall is high (>1100mm) in the western area of the forest, decreasing towards the eastern boundary (<700 mm) and the southern area near the coast has a longer wet season with higher rainfall than the northern area of the forest (Shearer and Tippett, 1989). Latitude and altitude determine a range of temperatures, with extremes of less than 0^{o}C in the south in winter to higher than 35^{o}C in the north in summer (Bureau of Meteorology; http://www.bom.gov.au/climate/austmaps/). However, not all years are seasonally consistent and extremes of rainfall and temperature can have implications for research and management (Chapter 5).

Vegetation

Flora of the jarrah forest has evolved, adapting to the environment. In the terminology of Specht (1969), the jarrah forest is dry sclerophyll, open forest. A more specialized description of site-vegetation types in the northern area of the jarrah forest, determined by topography, available moisture, soil acidity and soil fertility, was compiled by Havel (1975a and 1975b). Dominant in the canopy species is *Eucalyptus marginata* (jarrah) with *Corymbia calophylla* (marri) (formerly *Eucalyptus calophylla*), a co-dominant in some areas. In a floristic composition of ferns, cycads, conifers, monocots and dicots, it is estimated that more than 750 plant species are represented in the jarrah forest, and families most represented are the Proteaceae, Papilionaceae and Myrtaceae, all with more than 60 species present (Bell and Heddle, 1989). Terrestrial orchids are well represented with 51 species. *Xanthorrhoea* spp. are present in the understorey in some areas (Fig. 1.2). *Banksia grandis* (Proteaceae) (Fig. 1.2 inset), *Allocasuarina fraseriana* (Casuarinaeae) and *Persoonia longifolia* (Proteaceae) are the main components of the sub-canopy (Shearer and Tippett, 1989).

Eucalyptus marginata

E. marginata grows to 40 metres high and is usually straight boled, though mallee forms occur at the extremities of its distribution. Its rough, dark grey bark in long flat vertical strips is an identifiable feature (Brooker and Kleinig, 1990). Prized for its termite resistant and richly coloured, reddish timber, this slow-growing hardwood tree has been used extensively in furniture production and for floorboards. The history and controversy of commercial interests in the jarrah forest are reviewed in Schur (1985) and Havel (1989). The species name is derived from the distinctive margin outlining the outer leaf edge. While juvenile leaves can be sessile and ovate, the adult leaves become petiolate and lanceolate to falcate, growing 80 to 130 mm long (Brooker and Kleinig, 1990). Doley (1967) noted that leaves hung obliquely downwards with the upper surface away from the trunk. Stomata are located on the adaxial surface (Grieve, 1956; Doley, 1967). With molecular evidence adding to the morphological criteria for classification keys, *Eucalyptus* spp. have been reclassified into two groups, (1) Angophora which includes the genus *Corymbia* and (2) *Eucalyptus* with three sub-genera, *Eudesmia*, *Monocalyptus* and *Symphyomyrtus* (Ladiges, 1997). *Eucalyptus marginata* Donn ex Smith is included in *Monocalyptus.*

Figure 1.2 *Xanthorrhoea* spp. (grass trees) are a component of the jarrah (*Eucalyptus marginata*) forest, and are susceptible to *Phytophthora cinnamomi*.

Inset: The distinctive foliage of *Banksia grandis*, an understorey tree, which is highly susceptible to *P. cinnamomi*.

After the first year of growth, jarrah seedlings, like most eucalypts, develop a woody lignotuber, a swelling of the stem near, or just below, ground level. This adaptation, which originates as vegetative buds in the axils of the cotyledons and early leaves, has contributed to the species survival, as it has the ability to resprout after the tree has been damaged by frost, fire or harvesting (Brooker and Kleinig, 1990). The lignotubers of *E. marginata* may also be more resistant to pathogen attack than those of the co-dominant canopy species, *Corymbia calophylla* (Hardy *et al*., 1996). Reproduction of jarrah is initiated in spring and summer, when stalked buds form in clusters of seven to twenty-one and white flowers develop. The fruits are spherical to barrel-shaped, with diameters of nine to sixteen mm (Brooker and Kleinig, 1990).

E. marginata *and drought*

Drought has, for millennia, played an important role in the evolution of Australian flora and in the functioning of associated ecosystems. The dynamics of these processes are complex, with many other inter-relating biotic and abiotic factors making significant contributions. Generally, eucalypts and other sclerophyllous species have become drought-tolerant, with stomatal regulation of transpiration to conserve plant moisture and alignment of their vertical hanging leaves to avoid the evaporative effect of direct sunlight. However, *E. marginata* is an exception. Even during summer, these trees do not regulate the loss of water via stomata to a great extent (Grieve, 1956; Doley, 1967; Colquhoun *et al*., 1984). In the field, the transpirational requirements may be met by its extensive root system, able to access groundwater at depth (Grieve, 1956; Dell *et al*., 1983; Carbon *et al*., 1981).

1.2 *Phytophthora cinnamomi*

Early indications of disease in the jarrah forest

Deaths of *E. marginata* in the northern section of the forest during the 1920s were noted but not investigated. After the implementation of post-war roadwork projects in the late 1940s, an association of dead trees and understorey species with new roads became apparent. The deaths were unexplained by investigations comparing affected sites with healthy forest. Drought, nutritional deficiencies, waterlogging, toxicity and known pathogens were considered (Podger, 1968). The spread of the

problem, now referred to as jarrah dieback, was reaching epidemic proportions and affecting economic interests.

Isolation of P. cinnamomi *and its host range*

The agent responsible for the destruction was isolated from soil beneath dying *E. marginata* in 1964 by G.A. Zentmyer (Podger *et al*., 1965; Zentmyer, 1980). This was identified as *Phytophthora cinnamomi*, type A_2, and was the first record of the pathogen in Western Australia. Continued research confirmed that it was responsible for the death of *E. marginata* and other hosts in indigenous plant communities (Podger, 1972). *P. cinnamomi* was originally described in 1922 by Rands (cited by Zentmyer, 1980), who isolated it from cinnamon trees (*Cinnamomum burmanii*) in Sumatra, Indonesia. It was later identified as a major pathogen of hundreds of mostly dicotyledonous plants in over 60 countries, including commercial crops of avocado, chestnuts and ornamentals such as camellias and rhododendrons (Zentmyer, 1980). In Western Australia, it now affects over 2000 species, with the Proteaceae, Myrtaceae, Epacridaceae, Dilleniaceae families and the monocotyledonous Xanthorroeaceae especially susceptible (Wills, 1992). It is spread by groundwater (Shea *et al*., 1983; Kinal *et al*., 1993) and by human intervention when soil or infected plants are transported (Shearer and Tippett, 1989; Batini, 1992). Early research in Western Australia isolated *P. cinnamomi* from 55 species of plants growing in areas naturally affected by the syndrome known then as 'jarrah dieback' (Podger, 1968) and additions to its host range in the jarrah forest are still being made (McDougall *et al*., 2001). Within the eucalypts, the sub-genus *Monocalyptus* is more susceptible than other sub-genera (Podger and Batini, 1971; Marks *et al*., 1981; Tippett *et al*., 1985) though there is variation of susceptibility within species (Marks *et al*., 1981; Stukely and Crane, 1994).

Biology and survival of P. cinnamomi

Details of the biology and reproduction of *P. cinnamomi* are given by a number of researchers (Zentmyer, 1980; Erwin *et al*., 1983; Hardham *et al*., 1994). Commonly referred to as a fungus[1], *P. cinnamomi* is actually an Oomycete, a water mould, Class Oomycetes (Pythiaceae) and moisture is essential for its reproduction (Zentmyer, 1980;

[1] *P. cinnamomi* is placed within the Kingdom Chromista and is classed as an Oomycete, a water mould, but the commonly applied terms 'fungus' and 'fungal' will be used in the text of this thesis.

Duniway, 1983). Briefly, *P. cinnamomi* is a soilborne pathogen, infecting roots and collars of susceptible species when optimal conditions are met. Factors which influence the growth and survival of *P. cinnamomi*, particularly temperature, light, pH, aeration and moisture will be referred to in the relevant chapters in this study. It can reproduce asexually as mycelium, from which, when nutrients are limited, sporangia form. From these ovoid, non-papillate structures, motile, biflagellate zoospores are released. Chemotaxis was thought to attract the zoospores to the roots of host plants (Hickman, 1970; Carlile, 1983) where they encyst, adhere and germinate in the first stage of the infection process (Hardham, 2001), but recent research shows that, within the electrical fields generated by roots, motile zoospores are attracted to certain zones by electrotaxis (van West *et al*., 2002).

If both A_1 and A_2 mating types of *P. cinnamomi* combine, sexually produced oospores result. In the jarrah forest, most isolates recovered have been of A_2 mating type. (Shearer and Tippett, 1989). *P. cinnamomi* is usually heterothallic (Brasier, 1983), vegetatively producing chlamydospores, spherical thin-walled survival units occurring often on hyphal tips (Zentmyer, 1980). With these structures, it is able to survive for longer periods in adverse conditions. It can survive for up to 6 years in moist non-sterile soil, or for 80 days in dead or decaying plant tissue (Zentmyer and Mircetich, 1966), though assessments of its ability to survive in soil vary (Marks *et al*., 1975; Malajczuk, 1983). Depending on the number and type of competing microorganisms in the soil, *P. cinnamomi*, with both parasitic and saprophytic phases, has considerable potential to survive (Weste, 1983). Moisture and temperature are important factors in the survival of *P. cinnamomi* in soil (Nesbitt *et al*., 1979 b) and, depending on the soil type, it can also survive at depth where soil is not as prone to moisture loss as top soil and the fungus is less likely to encounter the antagonistic microbes that are more abundant in surface soil (Shearer and Shea, 1987). Even at matric potentials of –300 to –500 kPa, chlamydospores can survive for up to eight months in non-sterile soil without host tissue (Weste and Vithanage, 1979). Chlamydospores have also been observed in lesions of *E. marginata* and *P. cinnamomi* has been recovered from lesions in *E. marginata* roots, up to 2 years after inoculation (Tippett *et al*., 1985).

Ability to persist as a saprophyte in the necrotic tissue of colonized hosts is also indicated by the fact that *P. cinnamomi* has been isolated from dead bark and wood of *Banksia grandis*, an understorey species in the jarrah forest, after the trees have been dead for about one year (Shea, 1979) but it has been suggested that, after this time, its saprophytic phase declines (Blowes *et al*., 1982). However, the pathogen could be surviving as chlamydospores until conditions are favourable for reproduction and not relying on mycelial growth. In moist lateritic soil, it has been shown to survive in *E. marginata* root segments for 56 days (Old *et al*., 1984) but clearly has the potential for extended periods of survival, especially in jarrah forest soils, which have limited competing microorganisms (Podger, 1972). When soil was kept at container capacity, *P. cinnamomi* was recovered from mine site soil and jarrah forest soil after 210 days, but only after 112 days when soil was allowed to dry (Collins *et al*., 2001). In dried and re-moistened jarrah forest soil, *P. cinnamomi* survived for up to 18 months (Bunny, 1996). Initial dispersal is also favoured by the low levels of microbial activity. Sporangia can be induced by the action of soil microbes (Chee and Newhook, 1966). Soil microbes cause lysis of hyphae which triggers the formation of sporangia, but increasing amounts of microbial activity results in aborted development of sporangia (Nesbitt e*t al*., 1979a). In favourable field conditions, sporangia can release zoospores within 8 hours of adequate rainfall (Dr. G. Hardy, *pers. comm*.). The versatility of its survival strategy means that the threat of a subsequent outbreak of disease from infected hosts and from surviving propagules in these soils is high.

Centre of origin

The centre of origin of *P. cinnamomi* is thought to be within the tropical belt of forests in Indonesia or Malaysia, but there has been debate about its geographic origin and its introduction into Australia (Newhook and Podger, 1972; Pratt and Heather, 1973; Shepherd, 1975; Zentmyer, 1988). More recent molecular evidence, showing a lack of genetic variation in the isolates, indicates that it is not an indigenous pathogen of the south-west (Dobrowolski, 1999) or of the south-east of Australia (Old *et al*., 1988; Dobrowolski, 1999) nor of South Africa (Linde *et al*., 1999).

Cellular composition

P. cinnamomi, as described earlier, is a water mould, with characteristics which distinguish it from the fungi and align it more closely with algae. Though morphology and biochemical composition may vary during changes in the life cycle, cell walls of *Phytophthora* spp. contain no chitin, but are composed mainly of non-cellulosic and cellulosic β-glucans with proteins, lipids, sugars and other polysaccharides (Bartnicki-Garcia and Wang, 1983). The cellulosic content in the cell walls of *P. cinnamomi* decreases the effectiveness of histological stains normally used for chitinous fungi when infected plant tissue sections (also cellulosic) are examined for the presence of the pathogen. There is little distinction between host and pathogen in the stained sections. In studies of *P. cinnamomi* infecting *E. marginata*, O'Gara (1998) used several stains including Toluidine Blue O for polyphenolics, Sudan Black B for the detection of suberin and Phloroglucinol/HCl for lignin. Tippett *et al*., (1983) found that hyphae of *P. cinnamomi* were best observed with a Sudan IV-lactophenol stain. After initial experimentation with these stains, a combination of Saffranin and Fast green was used in the current study.

Pathogenicity and impact of P. cinnamomi *in forests*

The pathogenicity between isolates of *P. cinnamomi* varies and only one isolate (MU 94-48) described as highly virulent in jarrah (Hüberli, 1995) was used throughout this study. However, the ability of this pathogen to colonize and destroy host plants is well recognized. In a review of disease caused by *P. cinnamomi* in Australasian forests, Weste and Marks (1987) stated:

> "*There are no comparable records in the history of plant pathology of a pathogen invading native forests and destroying whole natural plant communities on the scale observed in Australia*."

Infection by this pathogen has now been officially recognized as a key threatening process to biodiversity in Australia in the Commonwealth's Environmental Protection and Biodiversity Conservation Act 1999.

1.3 *P. cinnamomi* and bauxite mining in the jarrah forest

Impact in mining rehabilitation

While much of the research into the impact of *P. cinnamomi* has centred on the plant communities of forested areas in the south-east and the south-west of Australia (Podger, 1968; Old, 1978; Weste and Marks, 1987; Davison and Tay, 1988; Shearer and Tippett, 1989), attention has also turned to rehabilitated mine sites (Colquhoun and Petersen, 1994; Hardy *et al*., 1996; Ward *et al*., 1996; O'Gara *et al*., 1997; Tynan *et al*., 2001). Within the jarrah forest of Western Australia, Alcoa World Alumina (Australia) mines for bauxite. Operations commenced in 1963 at Jarrahdale, where part of the current study (Chapter 5) was conducted, and that mine was closed in 1998. Other mines currently operate at Huntly and Willowdale. In the rehabilitation of these mined sites, revegetation can be hampered by the presence of *P. cinnamomi* in the soil, necessitating strict measures of hygiene to be imposed (Colquhoun and Hardy, 2000).

Differences between forest and mine site conditions

In the refilled, exposed mine pits, environmental conditions are different from the forest as a result of the lack of canopy. McChesney *et al*. (1995) reported that moisture of topsoil was lower and that temperature fluctuations were greater in open sites than in nearby canopied forest sites. The lack of canopy means not only is there less leaf litter for nutrient recycling (Ward and Koch, 1996), but also less protection from sun and wind. Microclimates in forest sites and in open mine sites differ (McChesney *et al*., 1995) and research involving the aetiology of the pathogen in an open mine site must consider those differences.

Mining operations and history

Detailed accounts of each stage of the clearing, mining and land rehabilitation process have been published (Nichols *et al*., 1985; Ward *et al*., 1996; Colquhoun and Hardy, 2000). Briefly, after commercial timber has been removed, and the area cleared, surface topsoil to a depth of about 10 cms is set aside to preserve its seedbank. Then the overburden, a layer of gravelly soil to 40 cms below the topsoil is stockpiled, separately. After the next layer of bauxite caprock or duricrust, is penetrated, the friable

bauxite below is accessed and transported from the site by large trucks. A pit with about a 5 metre wall and a compacted floor is left. Bulldozers reshape the surface and the pit is refilled with overburden and topsoil until the overall landscape resembles the original terrain. A bulldozer with a winged tyne rips the soil surface to a depth greater than 1 metre, resulting in contoured riplines which reduce erosion by controlling water flow. The subsurface ripping also aids the penetration by roots as plants become established in the area.

Resulting conditions

Aggregation of fine clay particles washing into the riplines of rehabilitated mine sites can retard infiltration of water into the soil. This, and any compacted surface soil has a damming effect which results in the ponding observed in riplines after heavy rainfall, providing conditions conducive to the reproduction of *P. cinnamomi* and transport of infective propagules. When the pathogen is present in the soil or the ponds, an outbreak of the disease and subsequent infection of the collars of plants can occur (Hardy *et al*., 1996; O'Gara *et al*., 1997). Lower branches are often also submerged in ripline ponds (Fig. 1.3).

Figure 1.3 Lower branches of *Eucalyptus marginata* submerged in ponded water in riplines at a rehabilitated mine site at Jarrahdale.

1.4 Previous research

Overview of historical research

Though *P. cinnamomi* is capable of infecting stem, leaves and fruit of host plants (Zentmyer, 1980), its recognition as primarily a soilborne pathogen has focused research on root systems. Since the original description by Rands in 1922, and since its impact on economic interests, scientific projects have examined many aspects of the biology of *P. cinnamomi*, of its interactions with host plants and of abiotic and biotic influences. A large body of literature has been presented over several decades from many countries and reviewed by several authors (Zentmyer, 1980: Erwin *et al*., 1983; Weste and Marks, 1987; Irwin *et al*., 1995; Cahill, 1995). Other publications have reviewed the intense investigation of the pathogen's effect in the jarrah forest of Western Australia (Newhook and Podger, 1972; Dell *et al*., 1989; Shearer and Tippett, 1989) where more recent research has focused on efforts to contain its spread, particularly with the use of phosphite (Shearer and Fairman, 1997; Pilbeam *et al*., 2000; Tynan *et al*., 2001, Wilkinson *et al*., 2001 and Hardy *et al*., 2001). While this is an effective method of control, there are indications that some *P. cinnamomi* isolates may develop resistance to phosphite (Dobrowolski *et al*., 2003). Recent studies using *Acacia pulchella* with the highly susceptible *Banksia grandis* in rehabilitated mine sites have shown an increased resistance in surrounding plants to *P. cinnamomi* and indicate the potential for biological control of the pathogen (D'Souza, 2001).

Natural and experimental infection methods

Necrotic tissue is usually evident as darkened lesions when *P. cinnamomi* infects the roots of host plants (Tippett *et al*., 1983; Shearer and Tippett, 1989). As the disease advances, xylem transport is limited, or completely interrupted, and the crown will deteriorate and other leaves become chlorotic (Shearer and Tippett, 1989). Surface lesions can appear on stems or branches, though some infected plants may not display symptoms (O'Gara *et al*., 1997; Hüberli *et al*., 2000). Depending on the progress of colonization by the pathogen, xylem function may be sufficient to allow the infected plant to survive for years, or so inadequate that the plant rapidly succumbs to the disease (Shearer and Tippett, 1989).

One aspect of research investigating the development of disease commonly involved the wound inoculation of root tissue with *P. cinnamomi* (Marks *et al*., 1981; Tippett *et al*., 1983). However, topographic, edaphic and environmental conditions in the open mine site are different to the adjacent undisturbed forest (McChesney *et al*., 1995). The aetiology of the disease is therefore likely to be different in the open mine site waiting to be revegetated to what it is in the adjacent non-disturbed forest sites (Dr. G. Hardy, *pers. comm*.). *E. marginata* seedlings have been shown to be more susceptible to infection by *P. cinnamomi* when waterlogged (Davison and Tay, 1985) but other experiments that simulating waterlogged conditions by creating hypoxia in aeroponics chambers, suggested that waterlogging/ponding did not always predispose plants to infection by zoospores (Burgess *et al.*, 1998). Shearer and Shea (1987) reported higher densities of inoculum and greater recovery of *P. cinnamomi* from low-lying, water gaining or irrigated areas compared to free-draining upland areas. The ponding of riplines after heavy rainfall is common in rehabilitated mine sites but does not necessarily indicate waterlogging of plants (Burgess *et al*., 1999b). While roots can become infected in mine sites, *P. cinnamomi* has been isolated from the collar and lignotuber of *E. marginata* without being present in the roots, leading to hypotheses that ponding in riplines not only predisposed plant tissue to infection but also provided an infection court for *P. cinnamomi* (Hardy *et al*., 1996). While previous inoculation techniques in forest studies have involved wounding of host tissue (Tippett *et al*., 1983; Shearer *et al*., 1987; Davison *et al*., 1994), a newly developed method (O'Gara *et al*., 1997) showed that infection can occur in the collar and stem of *E. marginata* without wounding, supporting those observations and hypotheses.

Stem inoculation does not mask the differences in susceptibility of a species to a pathogen, as root inoculation can (Tippett *et al*., 1985), and was the preferred site of inoculation in most experiments in this thesis (Chapters 3, 5, 6, 7 and 8). Also in the current study, further research was undertaken to develop an inoculation technique that avoided wounding and that was suitable for use in both the field and glasshouse (Chapter 4).

Host defence responses to wounding and infection

Plants synthesize many secondary metabolites, including phenolic compounds, in a defence response to wounding, herbivory and pathogenic attack (Rosenthal, 1986;

Bennett and Wallsgrove, 1994; Dixon *et al*., 1994, Pearce, 1996). Wounding during inoculation can confound interpretation of results as the pathogen may be responding to secondary metabolites produced by the host plant as a response to wounding. The non-wounding inoculation technique avoids the subsequent synthesis of extra phenolic compounds. The physiological and biochemical responses to wounding and to infection by pathogens varies between species and the complexity of the interactions and the influence of other factors, including drought and the timing of responses, must be addressed (Bostock and Stermer, 1989). Some phenolic compounds are a precursor to the production of lignin *in planta* and the lignification of cell walls, in cells immediately adjacent to wounded tissue can present a barrier, resistant to pathogenic invasion (Vance *et al*., 1980). This defence mechanism may not always be effective, if the rate of biosynthesis and deposition is too slow to contain the initial invasion (Bostock and Stermer, 1989; Biggs and Miles 1988; Bennett and Wallsgrove, 1994), and as the breakout of *P. cinnamomi* from previously confined lesions of *E. marginata* demonstrates (Tippett and Hill, 1983). Levels of phenolic compounds have been shown to vary between genotypes and *E. marginata* plants more resistant to *P. cinnamomi* will synthesize a higher proportion of phenolic compounds *de novo* in response to pathogenic invasion of tissue (Cahill *et al*., 1992).

Levels of the phytohormone, abscisic acid, rise after wounding and stimulate the biosynthesis of suberin, a polymer which is deposited in cell walls (Soliday *et al*., 1978). The hydrophobic waxes associated with suberin provide additional physical barriers and help to maintain the cellular integrity at the interface of cells and surrounding environment (Pearce 1996; Bostock and Stermer 1989). Suberin contributes to the compartmentalization of decay in trees (CODIT) (Shigo, 1984; Bostock and Stermer, 1989). In some eucalypts the accumulation of polyphenolic resinous gum (kino) forms a protective barrier after pathogenic attack or wounding (Marks *et al*., 1981) and it has been observed as an initial defence response by *E. marginata* after invasion by *P. cinnamomi* (Tippett *et al*., 1983). It has been suggested that these suberized or polyphenolic barrier zones are only indirectly providing a protection against pathogens, that their primary purpose is to seal wounds against water loss or the entry of air (Pearce, 1996). In the current study, veins of kino were observed in the field (Chapter 5) and sections of inoculated stem tissue were examined microscopically for evidence of increased levels of lignin and suberin (Chapter 3).

Recovery of the pathogen

For an accurate assessment of disease in an experiment or survey, it is essential to recover *P. cinnamomi* from all infected plants. Accurate monitoring of the pathogen is important to assess its distribution in field situations or to determine the susceptiblity of individual plants. Recoveries have been made from symptomless tissue and at times no recovery has been made from lesioned tissue (Crombie and Tippett, 1990; Hüberli *et al*., 2000). Researchers have noted a decline in recovery of the pathogen from infected plants over time (Davison *et al*., 1994; Duncan and Keane, 1996; O'Gara 1998; McDougall *et al.,* 2002). This lack of recovery from obviously diseased plants raises questions. Are the recovery techniques currently used adequate to assess the full extent of the spread of the pathogen? Has the pathogen perished when denied nutrients after its spread has been contained by the plant? Is the pathogen in a dormant state, as chlamydospores, and are the recovery techniques unable to break that dormancy?

Pythium spp. are often present in bark and can mask the presence of *Phytophthora* (Masago *et al*., 1977; Tsao and Guy, 1977), but the development of agars, selective for *Phytophthora*, has increased successful detection of the pathogen. The addition of antibiotics to a medium will suppress the growth of bacteria and antifungal compounds will prevent contamination by fungi, which may be present. *P. cinnamomi* was first isolated from *E. marginata* with avocado bait and antibiotics in agar (Zentmyer, 1980). A range of media, selective for *Phytophthora*, has been developed to detect its presence in plant tissue (Tsao and Ocana, 1969; Masago *et al*., 1977; Tsao and Guy, 1977; Kannewischer and Mitchell, 1981; Jeffers and Martin, 1986; Shearer and Dillon, 1995; Hüberli, 2000). Conversely, there is the problem of false positive recoveries of the pathogen. To avoid this, strict hygiene must be maintained to avoid cross-infection of plant samples. Using sterilized tools to cut segments of infected stem or roots firstly from a region further from the region of inoculation or infection site then cutting down to the more likely infected region will reduce the risk of cross-contamination when preparing samples for recovery. Surface sterilization of stem segments by dipping briefly in 70% ethanol, then either rinsing in sterile distilled water or flaming the segment to remove the ethanol, can eliminate surface contaminants, such as *Pythium* spp., without killing the *Phytophthora* resident in the phloem.

Advances in detection of soilborne pathogens have been reviewed by Cahill (1999) and include the use of dip-stick assays for detection of *P. cinnamomi* in soil (Cahill and Hardham, 1994) or in diseased tissue with monoclonal antibodies (Hardham *et al*., 1991; Gabor *et al*., 1993) and the trialling of various DNA extraction methods to use with PCR to detect fungal DNA in plant tissue and soil (Dobrowolski and O'Brien, 1993). However, standard mycological techniques, though slower, are less expensive and still have a place in research, especially to meet the needs of preliminary assessment.

Drought and expression of disease

Plants under environmental or nutritional stress are usually predisposed to disease (Schoeneweiss, 1975; Boyer, 1995) or infection may increase the detrimental effects of drought (Duniway 1976). The response can vary between species. Increased susceptibility to *P. cinnamomi* has been shown with cultivars of rhododendrons in droughted conditions (Blaker and MacDonald, 1981) but not in some species of oak (Robin *et al*., 2001). The development of lesions in *E. marginata* infected with *P. cinnamomi* has been shown to be less in plants with greater water deficit (Tippett and Hill, 1983; Bunny *et al*., 1995). Drought is a major factor in the Western Australian environment and *P. cinnamomi* a major pathogen, but apart from these studies, relatively little research has been made into the interaction of drought and infection of *E. marginata* by *P. cinnamomi*. Osmotic adjustment to increasing water deficit allows plants to become drought tolerant (Turner and Jones, 1980; Morgan, 1984). In the present study, the degree of tolerance of *E. marginata* seedlings to increasing water deficits was investigated (Chapter 2) prior to the establishment of glasshouse trials (Chapter 3, 6, 7 and 8).

Water stress in *E. marginata* has been recorded by several parameters, including (1) xylem pressure potential (Carbon *et al*., 1981; Colquhoun *et al*., 1984; O'Gara, 1998) using the pressure chamber (Scholander, 1965) and (2) stomatal conductance (Davison and Tay, 1985; Crombie and Tippett, 1990; Burgess *et al.*, 1999a) using a porometer and (3) relative water content (Tippett *et al*., 1987; Stoneman *et al*., 1994). These methods were used in the current study. Chlorophyll fluorescence has been used in some studies with other species (Mohammed *et al*., 1995). A range of osmotica e.g. polyethylene glycol (PEG) may be introduced into substrate media to simulate drought.

For the current study, use of PEG as an osmoticum was rejected because it can cause tissue damage (Fan and Blake, 1997). The phloem sugars in *E. globulus* and other eucalypts were measured to quantify water stress (Pate *et al*., 1998; Pate and Arthur, 1998). This method was trialled during the current study but, despite sampling in different seasons, stems of *E. marginata* did not yield exudate as readily as *E. globulus*.

Selection of genotypes resistant to P. cinnamomi

To enhance revegetation of mined sites and to restore forest ecoystems impoverished by the presence of *P. cinnamomi*, *E. marginata* genotypes resistant to *P. cinnamomi* have been selected (McComb *et al*., 1990). Seedling families were classified as resistant or susceptible from glasshouse tests with *P. cinnamomi*, then resistant or susceptible seedlings selected and cloned from these families (Stukely and Crane 1994). Field trials have confirmed the resistance or susceptibility of selected families (McComb *et al*., 1994). Higher levels of phenolics and higher concentrations of lignin were found in the tissue of resistant clonal *E. marginata* plants (Cahill *et al*., 1993). Seed orchards of resistant *E. marginata* plants have been established by Alcoa World Alumina (Australia) for revegetation of *P. cinnamomi*-affected forest (Dr. I. Colquhoun, *pers. comm*.). Experiments in the current study used both resistant and susceptible clonal lines of *E. marginata*, as well as seedlings.

1.5 The current study

Drought is a major abiotic stress with which the flora of the south-west of Western Australia has to contend. Disease caused by the introduction of *P. cinnamomi* is a major biotic stress. While indigenous plants have adapted to the climatic conditions and the recurring seasonal water deficits, they have not co-evolved with this pathogen and many species are susceptible. Relatively few studies have investigated the combined effect of both these major stressors on the vegetation. *P. cinnamomi* is believed to have evolved in tropical regions and needs warmth and moisture for reproduction and the infective phase of its life cycle. Timing of rainfall, especially unseasonally high summer rainfall, can be an important factor in the aetiology of disease caused by this pathogen.

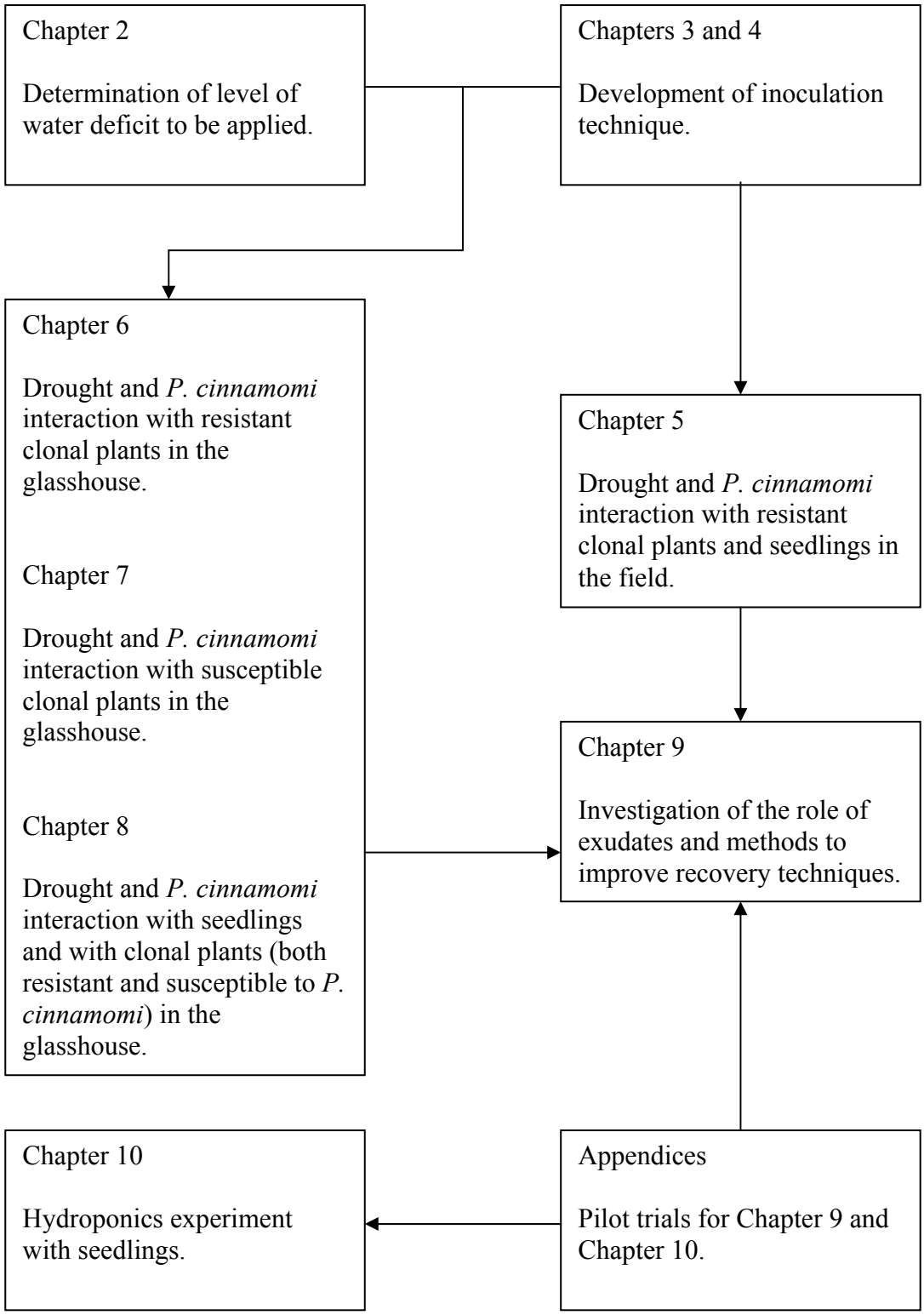


Figure 1.4 Flowchart of practical work undertaken during the current study.

Could drought be a surprising ally in some plants' defences against invasion by *P. cinnamomi* and the subsequent development of disease? In this study the effect of *P. cinnamomi* on *E. marginata* under different watering regimes was examined (Fig. 1.4). A consistent colour coding system (see Abbreviations) has been used throughout the thesis to indicate the watering regimes imposed.

The aim of this study was to investigate two hypotheses:

(1) Droughted *E. marginata* plants will show more resistance to *P. cinnamomi* than irrigated plants in the field and more resistance than plants grown at container capacity in the glasshouse.

(2) Drought stress will result in genotypes of *E. marginata* selected for resistance to *P. cinnamomi* showing more resistance to the pathogen than susceptible genotypes or seedlings.

Results obtained from the initial experiments led to two further hypotheses:

(3) Exudates from infected tissues of *E. marginata* plants reduce the probability of isolation of *P. cinnamomi* from such tissues.

(4) The addition of phenolic compounds to nutrient solution taken up by *E. marginata* seedlings will increase their resistance to *P. cinnamomi*.

Initial experiments were conducted to determine appropriate protocols for imposing simulated summer drought (Chapter 2) and for inoculating plants with *P. cinnamomi* in the field and in the glasshouse (Chapters 3 and 4). These protocols were applied in the field (Chapter 5) and in the glasshouse (Chapters 6, 7 and 8). In these experiments, *P. cinnamomi* was not always recovered from lesioned stems (Chapter 5) but was recovered from some symptomless tissue (Chapter 6). Methods to improve the accuracy of assessment of the presence of disease were found by rendering the inhibitory compounds in exudates from infected tissue less effective (Chapter 9). Finally, the results of an experiment, in which an increase was made in the level of a phenolic compound available for uptake by roots, suggested that this increase enhanced defence strategies initiated by the plants when confronted by a pathogen (Chapter 10).

Chapter 2

Survival of *Eucalyptus marginata* (jarrah) seedlings at water deficits close to wilting point.

2.1 Introduction

Drought is a major factor governing plant survival and growth in the Australian environment. In the mediterranean climate of the south-west of Western Australia, the indigenous flora, including *Eucalyptus marginata* (jarrah), has adapted to the water deficits imposed on it by the six months of diminished rainfall from November to April during long, hot dry summers (Doley, 1967; Carbon *et al*., 1981). Susceptibility of plants to pathogenic attack may be greater when the host plant is subjected to stress (Schoeneweiss, 1975). There are exceptions and one may be the development of the disease caused by *Phytophthora cinnamomi* in its hosts. *P. cinnamomi* needs water to reproduce (Zentmyer, 1980) and it may be inhibited if the otherwise healthy host is stressed by a water deficit. In previous studies, inhibition of growth of *P. cinnamomi* lesions in *E. marginata* was noted when xylem pressure potential was greater, i.e. when trees were more stressed (Tippett *et al*., 1987), and the recovery of *P. cinnamomi* was lower from *E. marginata* trees experiencing water stress in a study of lesion development in relation to rainfall (Bunny *et al*., 1995). The current trial provided an indication of the response of healthy, non-inoculated seedlings to increasing water deficit and of their ability to survive by adjusting osmotically to simulated drought. This information was required for glasshouse experiments reported in Chapters 3, 6, 7 and 8 where the interaction of watering regimes, host susceptibility and development of disease caused *by P. cinnamomi* was studied.

The aims of this trial were (1) to determine the number of days it would take healthy *E. marginata* seedlings to reach wilting point from container capacity once water was withheld from substrates and (2) to determine the ability of the seedlings to survive at different levels of water deficit, when substrate water was partially restored.

2.2 Methods

2.2.1 *Experimental design*

In a repeated measures design, 10 nine-month-old *E. marginata* seedlings had increasing water deficits imposed on them after being droughted to four successive wilting points (WP 1 to WP 4) over a period of 10 weeks (Table 2.1; Fig. 2.1). Five other seedlings were maintained at container capacity as controls.

Table 2.1 Protocol for the imposition of increasing water deficit to determine the survival ability of ten *Eucalyptus marginata* seedlings.

Following	% of water loss restored to substrate *	Number of days the water deficit was maintained
Wilting point 1	30	8
Wilting point 2	20	8
Wilting point 3	10	8
Wilting point 4	5	14

* Water loss calculated as weight of replicate at container capacity less weight at wilting point.

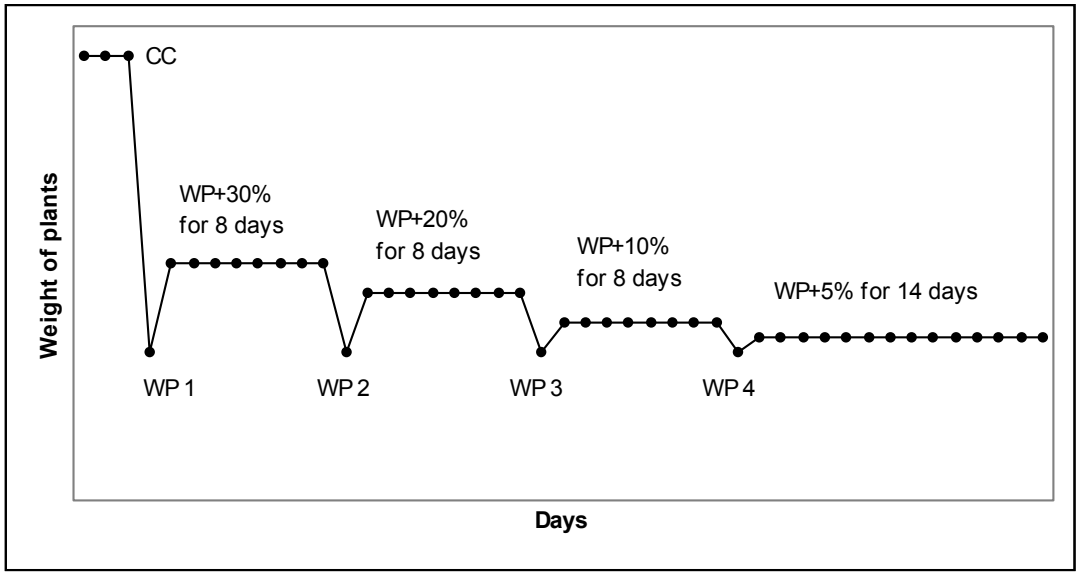


Figure 2.1 Schematic diagram of experimental design, showing hypothetical, relative weight with increasing water deficit of the 10 *E. marginata* seedlings, from container capacity (CC) to each successive wilting point (WP) and the restoration of a percentage of water lost from CC to WP.

2.2.2 *Plant material and growing conditions*

Six-month-old tubestock seedlings of *E. marginata* were obtained from the Alcoa World Alumina nursery at Marrinup and repotted into sterilized 150 mm free-draining polyvinyl containers in a standard sterilized peat/perlite (v:v 3:2) substrate with nutrients added (Appendix 1). The seedlings were staked and 5g of low-P slow release fertilizer (Osmocote, Scotts Australia Pty. Ltd. Cecil Avenue, Castle Hill NSW, 2154) was added to the substrate surface. They were maintained for 12 weeks in a 75% shade house and watered daily for 20 minutes by an overhead watering system.

Fifteen of these nine-month-old seedlings, ranging in height from 40 cm to 58 cm were selected and transferred to an evaporatively cooled and heated glasshouse. Mean stem diameter was 4.17 ± 0.26 mm. Seedling heights were measured at the start (Height 1) and end (Height 2) of the trial. Relative growth of control plants was compared to that of droughted seedlings (Equation 2.1).

Equation 2.1: Relative plant growth = $\frac{\text{Height 2} - \text{Height 1}}{\text{Height 1}} / \text{days to harvest}$

After being numbered 1-15 at random, all pots with plants were stood in bins of water until capillary action brought each substrate to saturation. During this time they were covered with plastic bags to minimize transpiration. After drainage of excess water, weights at container capacity (CC) were noted for all plants in containers. All plants were maintained at CC for two days. Five seedlings were kept at CC, with hand watering twice daily, throughout the trial as controls, and ten seedlings were subjected to simulated drought conditions by withholding water and allowing the substrate to dry until wilting point was reached. All plants were weighed daily. Weight in this trial refers to the weight of container, plant, substrate and water content. Evaporation rate of moisture in soil was noted by observing the decreasing weight of two similar containers of the peat/perlite mix (without plants) as they dried from CC to a stable weight. They were then placed in an oven at 60^{o}C for 7 days until they reached dry weight. The rate of evaporation was compared to the rate of evaporation and transpiration of all seedlings until the first wilting point (WP 1). The number of leaves and leaf lengths of each seedling were recorded at the start of the trial. The trial was conducted in the autumn 1999, from March to May. Maximum and minimum temperatures were recorded in the glasshouse each day.

2.2.3 *Levels of water deficit*

Water was withheld from the substrate of the 10 seedlings to be droughted after they had been maintained at CC for 2 days. The number of days taken by each plant to reach wilting point (WP) and the weight at WP were noted. The WP was determined when the tips of new growth were no longer turgid. When WP1 was reached, the youngest fully expanded leaf was excised at the stem to provide the greatest length of petiole. The xylem pressure potential was read using the Pressure Chamber method of Scholander *et al*. (1965). When seedlings reached WP1, substrate water content was replenished by 30% of the loss from CC to WP. The weight at which they were maintained (W_M) for 8 consecutive days, was calculated by the weight at WP (W_{WP}) plus 30% of the difference between the weight at CC (W_{CC}) and the weight at WP (Equation 2.2).

Equation 2.2: $$W_M = W_{WP} + 30\%(W_{CC} - W_{WP})$$

After WP was recorded, drainage holes in the base of the container were covered and sealed with silver ducting tape to retain the added water. After weights were maintained at $W_{WP} + 30\%(W_{CC} - W_{WP})$ for 8 days, water was again withheld from containers until wilting point was observed again (WP2). Water was then added to replace 20% of the water loss from CC to WP2 and this level maintained for 8 days. Water was withheld again and the procedure repeated, with daily weighings at levels of 10% and 5% replenishment, after WP3 and WP4 respectively, had been reached. Substrates were maintained at 5% replenishment for 14 days, after which time the trial was terminated (Table 2.1).

2.2.4 *Statistical analysis*

Data were analysed using Statistica® for Windows Version 5 (StatSoft Inc., OK, USA) and Excel for Windows (Microsoft Corporation, USA), Microsoft Excel Analysis ToolPak (GreyMatter International, Inc., Cambridge, MA 02141). Prior to parametric analyses, data were screened for basic assumptions of parametric tests including normal distribution, equality of variances and non-correlation of means and variances. In cases where data did not conform to assumptions, log transformations

were used to correct the deviations. The use of such transformations is noted in the relevant results. Where data were log-transformed, means are presented graphically for non-transformed data. After comparison of data between or within treatments, where an ANOVA resulted in a P-value of < 0.05, data were considered significantly different.

2.3 Results

2.3.1 *Plant growth and temperatures in the glasshouse*

There was no significant (*df* 1,13; P>0.05) difference in heights of plants between treatments at the start of the trial (Fig. 2.2). Increase in the mean height of control plants, maintained at CC throughout the trial, was greater than that of the droughted plants with a 58.8% and a 41.1% increase, respectively. A one-way ANOVA showed that there was a significant (*df* 1,13; P=0.02) difference between the increase in heights of control plants, not subjected to water deficit (n = 5), and of droughted plants (n = 10), from the start (Height 1) to the end of the experiment (Height 2) (Fig. 2.2). However, there was no significant (*df* 1,13; P=0.08) difference in the relative growth (Equation 2.1) between control plants and droughted seedlings.

There was no significant (P>0.05) difference in leaf length between control plants (mean 8.30±SE 0.79 cm) and plants to be droughted (mean 7.97±SE 0.26 cm) at the start but, at the end of the trial, leaves of control plants were visibly larger than the few surviving and mostly chlorotic leaves on the droughted plants (Fig 2.3). No measurements of leaf length were taken at the end of the experiment. Leaves had been excised for XPP and some had fallen due to droughted conditions. Temperatures in the glasshouse ranged from 13.2^{o}C to 37.5^{o}C during the trial (Table 2.2.)

Table 2.2 Temperatures in the glasshouse (March to May 1999).

	Maximum Temperatures ^{o}C	Minimum Temperatures ^{o}C	Number of days
from start to WP 1	25.1 – 32.8	13.2 – 20.1	14
WP 1 to WP 2	26.1 – 34.3	13.6 – 20.1	16
WP 2 to WP 3	21.2 – 37.5	14.8 – 20.4	13
WP 3 to WP 4	24.0 – 30.7	13.2 – 18.2	16
WP 4 to end	21.7 – 26.5	13.4 – 14.2	14

WP = Wilting point (when all seedlings have reached WP). Number of days includes days maintained at water deficit level and days water withheld until all seedlings reach next WP.

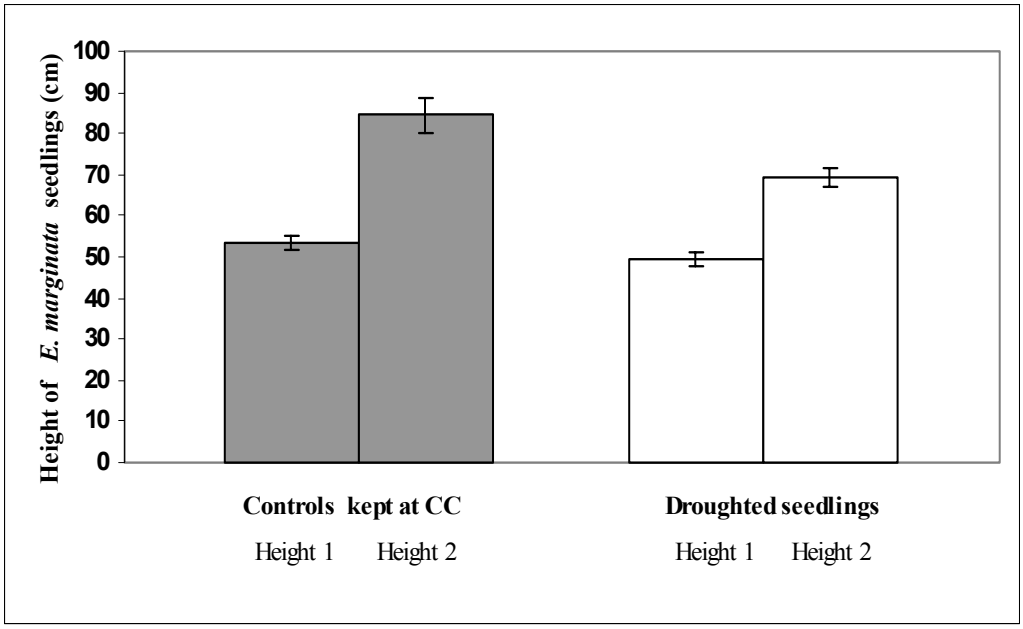


Figure 2.2 Mean heights of *E. marginata* seedlings kept at container capacity (CC) and of droughted seedlings. Height 1 = mean height of seedlings at start of trial. Height 2 = mean height of seedlings at end of trial, 10 weeks later.

Figure 2.3 Comparison of growth and condition of a representative control seedling (left) and a droughted seedling (right) at the end of the trial.

2.3.2 *Levels of water deficit*

Seedlings reached WP1 from 8 to 14 days after water was withheld. A repeated measures analysis showed a significant (*df* 3,24; P<0.001) difference in the mean weight of replicates at each WP which was progressively lower from WP1 to WP4 with imposition of greater water deficit (Fig. 2.4). With one exception, weight of individual seedlings was also progressively lower. All seedlings survived at the lowest moisture level (replenishment of 5% of the moisture loss to substrate) for 14 days, after which the trial was terminated. Xylem pressure potential, measured at WP 1, ranged from -1.42 MPa to > -3.5 MPa.

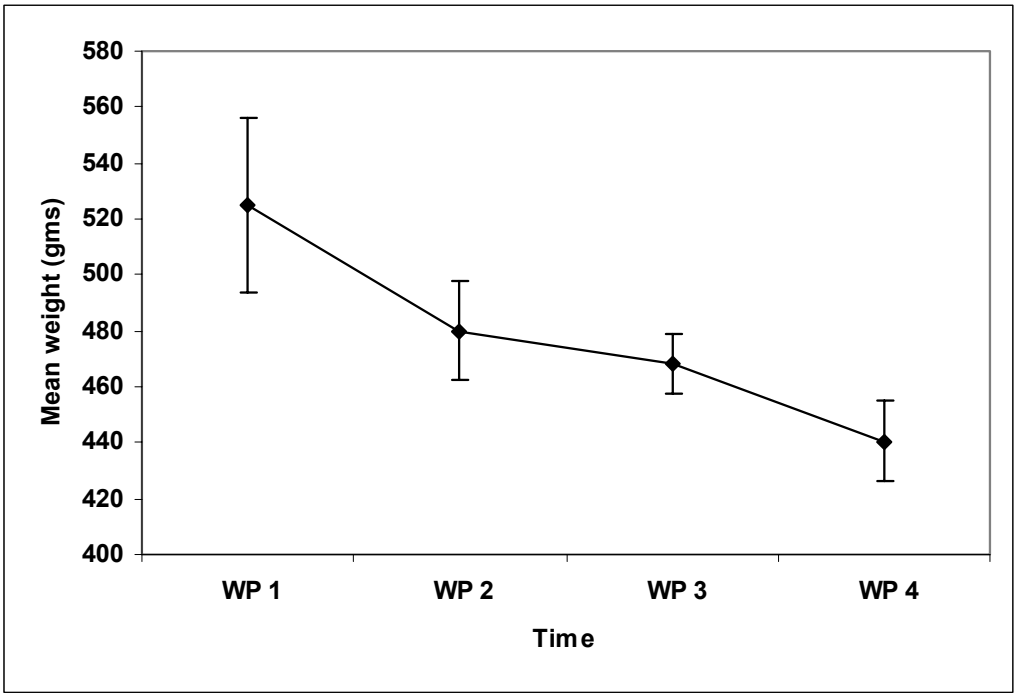


Figure 2.4 Progressively lower mean weight of plants and substrates at successive wilting points (WP1 to WP4) of 10 droughted *E. marginata* seedlings over 10 weeks.

Table 2.3 Days taken for *E. marginata* seedlings to reach wilting point at each level of increasing water deficit.

Number of replicates	Days to WP after water withheld	Maintained at water deficit of	Number of days maintained	Number of plants surviving
10	8 – 14	WP + 30%	8	10
10	5 - 8	WP + 20%	8	10
10	3 - 5	WP + 10%	8	10
10	5 - 8	WP + 5%	14	10

The number of leaves on droughted seedlings ranged from 14 to 37. Though the droughted seedling with the most leaves reached WP1 after 8 days and that with the least leaves after 14 days, there was no strong correlation between the number of days to WP1 and either (1) Height 1 of the seedlings (r = -0.25), (2) number of leaves (r = -0.6434) or (3) weight at CC (r = 0.24). The moisture loss from each replicate (combined evaporation and transpiration) was greater than the evaporation from the substrates without seedlings (Fig. 2.5).

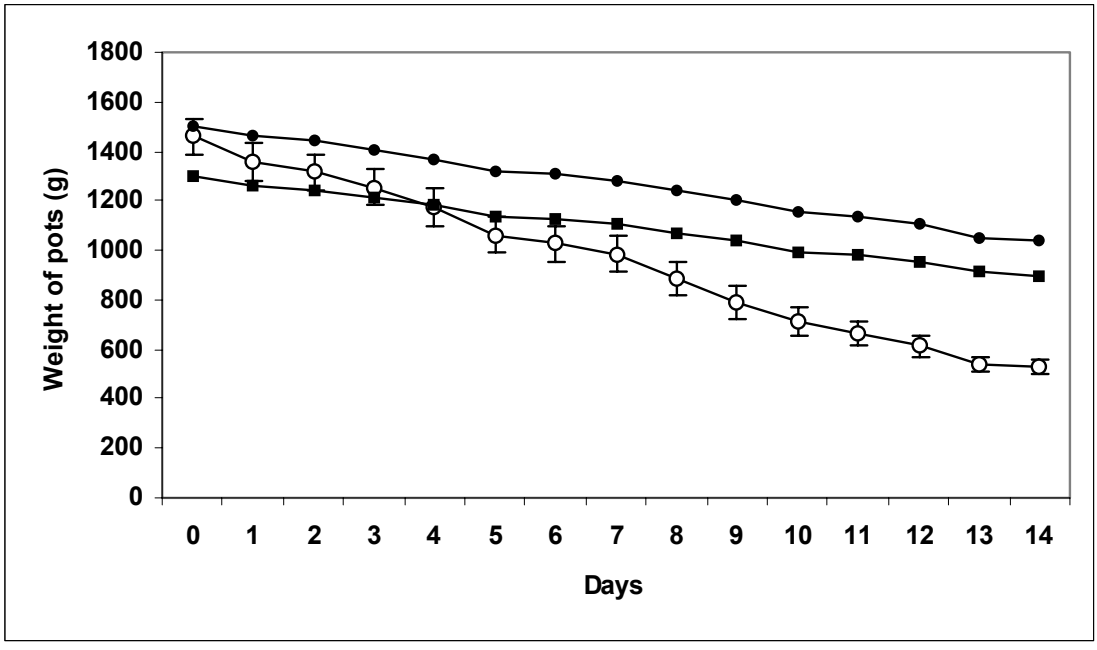


Figure 2.5 Comparison of the rate of moisture loss by evaporation from substrate of two pots without seedlings (black lines) and the loss from mean combined evaporation and transpiration of last 3 seedlings to reach wilting point (WP1) at 14 days (—o—). Bars represent the standard error of the mean.

2.4 Discussion

2.4.1 *Seedling survival*

Droughted *E. marginata* seedlings were able to survive at low moisture levels, and to adjust to progressively greater water deficits as demonstrated by the progressively lower wilting points. The anomaly of one seedling which had a higher weight at WP2 than WP1 is probably due to the subjectivity of using turgidity of tips of new growth as the criterion for determination of WP.

The ability of plants to maintain turgor when subjected to water stress has been noted in previous studies (Turner and Jones, 1980; Morgan, 1984). Slower imposition of water deficit results in greater osmotic adjustment or the ability to survive higher water deficits (Turner and Jones, 1980). In pot-grown seedlings of three other *Eucalyptus* spp., Myers and Neales (1986) found that drought induced osmotic adjustment and observed a greater (more negative) xylem pressure potential (XPP) after the first wilting. While XPP was not measured at each WP in the current study, because of the limited number of leaves at the start and leaf fall due to drought, the progressively lower mean weights at each successive WP (Fig. 2.4) indicated that *E. marginata* also responded by adjusting to droughted conditions.

The height and the weight of the replicate which reached WP1 first, was slightly greater than the mean and no correlation was found between the number of days to WP1 and any other observation. Leaf growth was less in droughted seedlings (Fig. 2.3). Stoneman *et al*. (1994) also found a reduced rate of leaf growth in droughted *E. marginata* seedlings (with some compensation after re-watering) where there was a linear relationship between the decrease in predawn water potential and decrease in leaf growth.

While some eucalypts conserve moisture by reducing transpiration in hot, dry conditions, *E. marginata* trees, with extensive roots able to access water at depth in field conditions (Dell *et al*., 1983), do not regulate water loss to the same extent (Doley, 1967), and continue to transpire at a higher rate than *Corymbia calophylla* (Grieve, 1956; Colquhoun *et al*., 1984). Though the prepared soil was mixed as uniformly as possible, containers were the same size and seedlings the same age, variance in soil volume, root mass and leaf area of plants in each container would account for

differences in soil water capacity, water uptake and rates of transpiration. While these factors affected the water loss in individual replicates, results indicated that *E. marginata* seedlings are capable of surviving under a high level of water stress.

2.4.2 *Conclusion*

This trial indicated that the nine-month-old seedlings could withstand water deficit, surviving at moisture levels as low as a replenishment of 5% of moisture loss after reaching WP. As a result of these findings, it was decided to apply a simulated droughting regime of replenishment of 10% of the moisture loss from CC to WP, when *E. marginata* plants in future experiments (Chapters 3, 6, 7 and 8) had reached WP, and to maintain them at that level for observations. The choice of 10% rather than 5% replenishment was to compensate for increased uptake and transpiration in older seedlings and for the increased stress imposed after inoculation with *P. cinnamomi*.

Chapter 3

Comparison of two techniques used to inoculate *Eucalyptus marginata* (jarrah) seedlings with *Phytophthora cinnamomi* under different watering regimes.

3.1 Introduction

The long, hot, dry summer normally experienced in the south-west of Western Australia is a factor in the aetiology of *Phytophthora cinnamomi* infecting *Eucalyptus marginata* in the forests and in the rehabilitated bauxite mine sites. Infection of susceptible forest eucalypts by *P. cinnamomi* was thought to occur through the fine roots until it was shown that major roots and collars also became infected using a shallow wounding technique (Marks *et al*., 1981). Stem or coppice stem inoculation of *E. marginata* in most previous field studies involved wounding of the host before the insertion of inoculum for the infection process (Tippett *et al*., 1983; Shearer *et al*., 1987; Davison *et al*., 1994; Bunny *et al*., 1995). Wounding methods result in successful inoculation when the pathogen is introduced directly into the phloem, but a wounded plant synthesizes phenolic compounds that lignify surrounding tissues and consequently the pathogen may be contained (Vance *et al*., 1980; Nicholson and Hammerschmidt, 1992).

Inoculation without extreme wounding by incision into the phloem is closer to the natural infection process. Zentmyer (1960) tested several methods when inoculating macadamia seedlings with *P. cinnamomi*, and as his non-wounding method failed to produce lesions, he suggested that a wound was necessary for successful infection. This accepted tenet was clearly disproved when O'Gara *et al*. (1996) demonstrated that, when stems were flooded, zoospores of *P. cinnamomi* could penetrate the unwounded periderm of *E. marginata* seedlings and colonize stem tissue. The technique was designed to simulate the ponding of riplines after heavy rain in rehabilitated mine sites, but it had not been tested on plants that had experienced severe drought conditions, followed by abundant rainfall. To improve rehabilitation strategies, a better understanding is required of the survival and reproduction of *P. cinnamomi* in mine sites, where factors different from those in a forest play a role. A large field trial was designed, to observe the expression of disease caused by *P. cinnamomi* in *E. marginata*

under different watering regimes in a rehabilitated mine site (Chapter 5). The trial required the inoculation of hundreds of plants, which would be subjected to severe summer drought, so a fast, simple, effective technique was required.

The current study was undertaken to determine the best inoculation method for the planned field trial (Chapter 5). The non-wounding method (O'Gara *et al*., 1996) and an underbark inoculation method (Davison *et al*., 1994) were assessed in a glasshouse experiment. Different watering regimes were imposed (Fig. 3.3) using information from a previous study (Chapter 2), that determined the level of water deficit in the substrate, at which *E. marginata* seedlings could survive. This level was maintained in the substrate of some plants while others were kept at container capacity. Some droughted plants were later restored to container capacity, simulating rainfall after a period of drought. The experimental design allowed comparison of the development of the disease caused by *P. cinnamomi* in droughted and non-droughted *E. marginata* seedlings.

The aims of this experiment were to compare a non-wounding inoculation technique with an underbark inoculation technique and to evaluate the effectiveness of each method, under different watering regimes, when inoculating stems of *E. marginata* seedlings with *P. cinnamomi*.

Three hypotheses were tested in this experiment.

H_o: There is no difference between the two inoculation techniques (underbark or zoospore inoculation), either in plant mortality, or the extent of colonization by *P. cinnamomi* in *E. marginata* seedlings.

H_o: The three watering regimes imposed have no effect on the mortality or the extent of colonization in *E. marginata* seedlings inoculated with *P. cinnamomi.*

H_o: Either the underbark or zoospore inoculation technique is appropriate for a large scale field trial.

3.2 Methods

3.2.1 *Experimental design*

The trial was a completely randomised block design consisting of two inoculation techniques and three watering regimes (Table 3.1). There were 16 replicates per treatment and 8 replicates in all controls with an equal proportion of all treatments and controls on each bench.

Table 3.1 Protocol for inoculation of *E. marginata* seedlings with *P. cinnamomi*. Two different stem inoculation methods were employed (a non-wounding method with zoospores of *P. cinnamomi* and an underbark method which wounded the stem). Three watering regimes were implemented: (1) substrates of seedlings were kept at container capacity (CC); (2) seedlings were droughted to wilting point (WP), then 10% of moisture loss from CC to WP was replaced and the substrate maintained at that level until harvest; (3) as (2), but after 2 weeks at the droughted level, moisture content of substrate was restored to CC.

Watering regime	Underbark inoculation		Zoospore inoculation	
	Inoculated *n* =	Control *n* =	Inoculated *n* =	Control *n* =
1	16	8	16	8
2	16	8	16	8
3	16	8	16	8

Watering regimes were as illustrated in Figure 3.3.
Control plants were sham inoculated with sterile distilled water or with sterile Miracloth™ discs. *n* = number of replicates.

Table 3.2 Revised protocol for the harvesting of *E. marginata* seedlings underbark-inoculated and zoospore-inoculated with *P. cinnamomi*.

	Harvest 1				Harvest 2	
	Underbark-inoculated		Zoospore-inoculated		Zoospore-inoculated	
Watering regime	Inoculated *n* =	Control *n* =	Inoculated *n* =	Control *n* =	Inoculated *n* =	Control *n* =
1	16	8	7	1	9	7
2	32	16	14	2	9	7
3	n/a	n/a	n/a	n/a	9	7

Harvest 1, 35 days after inoculation, included all surviving underbark-inoculated seedlings and those which had died prior to harvest, also zoospore-inoculated seedlings which had died or were not required for the continuation of the experiment.
Harvest 2, 63 days after inoculation, included all remaining zoospore-inoculated seedlings. *n* = number of replicates. n/a = not applicable. Control plants were sham inoculated with sterile distilled water or with sterile Miracloth™ discs.
Watering regimes 1, 2 and 3 are illustrated in Figure 3.3.

The high rate of mortality in underbark-inoculated seedlings prior to Harvest 1, 35 days after inoculation (Fig. 3.4), before watering regime 3 (Table 3.1) could be imposed, necessitated the rearrangement of surviving zoospore-inoculated seedlings to the 3 different watering regimes (Table 3.2). These seedlings were harvested 63 days after inoculation (Harvest 2).

3.2.2 *Plant material and growing conditions*

Six-month old *E. marginata* seedlings, provided by the Marrinup nursery of Alcoa World Alumina Australia, were grown for a further 6 months in 150 mm polyvinyl free-draining containers in a peat/perlite substrate (2:1, v:v) with added nutrients (Appendix 1). Plants were about 60 cm high and all had a naturally formed periderm up to 12 cm above the soil line. Lower leaves and branches were removed to allow easy access to the stem region to be inoculated and all plants were staked. The plants were then placed in an evaporatively cooled glasshouse to acclimatize for 3 weeks prior to the trial. Maximum temperatures in the glasshouse ranged from 19.1^{o}C to 30.5^{o}C and minima from 12.5^{o}C to 19.8^{o}C. After being given an application of Osmocote™ low phosphorus (Scotts Australia Pty. Ltd., 89 Cecil Avenue, Castle Hill NSW 2154), a slow release fertiliser, all plants were hand-watered twice daily to container capacity of the substrate. Stem widths were measured at the start of the experiment. Plant heights were measured at the start of the experiment and at harvest and relative plant growth calculated (Equation 3.1).

Equation 3.1: Relative plant growth = $\frac{(\text{Height 2} - \text{Height 1}) / \text{days to harvest}}{\text{Height 1}}$

3.2.3 P. cinnamomi *isolate*

Using the underbark inoculation technique, an isolate of *P. cinnamomi* (MU 94-48), collected from Willowdale in the south-west of Western Australia and highly virulent in *E. marginata* (Hüberli, 1995) was re-passaged through the stem of an *E. marginata* seedling to ensure that it was still aggressive (Appendix 9). When the seedling was harvested, 1 cm segments of infected stem were cut longitudinally, then plated onto sterile NARPH agar, selective for *Phytophthora* (Hüberli *et al*., 2000), in 90 mm Petri dishes. The plates were sealed with Parafilm™ and incubated at 24±1^{o}C in the dark for 5 days. The resulting outgrowth of the pathogen was sub-cultured again

onto NARPH selective agar then onto sterile V8 nutrient agar, (V8 Juice, Campbell's Soups Australia, Lemnos, Victoria. 3631) (Appendix 2a), to obtain an axenic culture of *P. cinnamomi* to be used in the preparation of inoculum.

3.2.4 *Preparation of zoospores*

Mycelium of *P. cinnamomi* was grown on V8 agar in 90 mm plastic Petri dishes for 5 days. Aseptically, from the actively growing colony margin, 1 cm x 1 cm mycelial mats were cut. These were transferred into 90 mm sterile glass Petri dishes and covered with 15 ml sterile V8 broth, pH 6.0 (Appendix 2b). All water used in aseptic procedures was sterile distilled water (SDW). Thirty plates with ten mats in each were incubated for 40 hours in a controlled temperature room at 24±2^{o}C under fluorescent light. The V8 broth was then syringed from the plates and the mats rinsed 3 times with SDW. The V8 broth was replaced with 15 ml of non-sterile soil filtrate (100g soil/litre distilled water; Dr. G. Hardy, *pers. comm.)* pH 6.0, which had been stirred for 3 hours then filtered through Whatman's paper No.1). A potting mix containing coarse river sand, fine pine bark and coconut peat (2:2:1; v:v:v) was used to produce the soil filtrate. Developing sporangia were observed with a compound microscope at 100x magnification (Olympus BH 2), 24 hours later, and plates were left under lights for another 24 hours at 24±2^{o}C to allow sporangia to mature.

To induce zoospore release from the sporangia, the culture was cold shocked at 4^{o}C for 30 minutes, then placed in an incubator at 24±1^{o}C for an hour, during which continual observations were made with the microscope to determine when optimal zoospore release had occurred (Appendix 5). When a high concentration of motile zoospores was seen, the solution was transferred to an acid-washed beaker in preparation for inoculation of the *E. marginata* plants. Glass beakers, measuring cylinders and Petri dishes used in the production of zoospores were immersed in 2M HCl for 24 hours then rinsed with sterile distilled water prior to use, to discourage encystment and attachment to the surfaces (Byrt and Grant, 1979). Five 1 µl drops of zoospore suspension were transferred to a V8 agar plate using a pipette with acid washed tips. Zoospores in each drop were counted, using the compound microscope at 200x magnification, and, by extrapolating this count, the remaining suspension was diluted by an appropriate volume of water in an acid washed beaker to yield a concentration of about 3000 zoospores L^{-1}. Plants were inoculated immediately.

3.2.5 *Preparation of Miracloth discs*

A paper punch was used to cut 6 mm diameter discs from a sheet of Miracloth™ (Calbiochem Corporation, La Jolla, CA, USA). After being autoclaved at 121°C for 20 minutes on three consecutive days in distilled water to eliminate bacterial contamination, the discs were placed around the perimeter of 90 mm Petri dishes containing V8 agar. Under aseptic conditions, an agar plug colonized with *P. cinnamomi* was placed in the centre of each plate. Plates were then sealed with Parafilm™ (American National Can, Greenwich, Ct. 06836) and incubated for 6 days in the dark at 24±1°C by which time the pathogen had colonized the Miracloth discs and they were ready to be used in the inoculation process.

3.2.6 *Inoculation processes*

(a) Zoospore suspension

A receptacle, as described by O'Gara *et al.* (1996), was constructed around the stems of plants which were to be inoculated with zoospores (Fig. 3.1). This consisted of a clear plastic, disposable 250 ml drinking cup, cut around the circumference about 5 cms from the base. The top portion was discarded. In a method slightly modified from O'Gara *et al.*, (1996), a heated cork borer was used to make an 8 mm hole in the base of the cup with the hole's diameter approximating that of the seedling's stem. Another cut was made down the side of the receptacle and across to the hole in the base. The stem was prepared with the 1 cm region of inoculation (ROI) in an internodal area, usually 4-8 cms above the soil. Directly below the intended ROI, Parafilm was wound around the stem. Over the Parafilm, a ring of Blu-Tack™ (Bostik (Australia) Pty. Ltd., Thomastown, Vic. 3074) encircled the stem. The receptacle was positioned so that it rested on and adhered to the Blu-Tack, and was sealed to the stem. One strip of silver ducting tape was used to align the cut edges and another covered the cut in the base, extended around the elbow and up the outside of the receptacle (Fig. 3.1). Additional plugs of Blu-Tack were pushed into areas where the potential to leak was greatest – the cut elbow area of the cup and the join at the stem. Zoospore contact with the Blu-Tack was minimal and a preliminary test showed that zoospore motility was not affected. To test for leakage, inoculum receptacles were attached to the stems of plants, which were

to be inoculated with zoospores, ten days prior to inoculation then left filled with SDW overnight.

The ten-day period also allowed recovery from any minor wounding inflicted during the attachment of the receptacles. All receptacles were again filled with SDW for 24 hours prior to inoculation when the SDW was removed with a syringe and receptacles on plants to be inoculated were filled immediately with the prepared zoospore suspension (Section 3.2.4) to a depth of 1 cm. The centimetre of stem in contact with the zoospore solution was designated the region of inoculation (ROI) of the seedling. Receptacles on control plants were refilled with SDW to a depth of 1 cm in a sham inoculation. The temperature of water in receptacles attached to stems of control plants was 21^{o}C to 22^{o}C at the time of inoculation. Receptacles were removed after 5 days.

(a) Underbark inoculation

An incision was made in an internodal area 4-8 cms from the base of the stem of those plants to be inoculated with the underbark method (Davison *et al*., 1994). This upward cut with a sterile razor blade extended into the phloem. Using sterile forceps, a Miracloth disc colonized with *P. cinnamomi* was inserted into this wound (Fig. 3.2). Sterile Miracloth discs were substituted for colonized discs in stems of control plants. A strip of Parafilm was wrapped around the stem over the wound to minimize entry of other pathogens and desiccation. A wrap of flagging tape, of a different colour for each treatment, gave further protection at the ROI. Surplus colonized discs were plated onto NARPH agar to confirm viability of the *P. cinnamomi* inoculum after exposure to glasshouse conditions.

Figure 3.1 Receptacle containing *P. cinnamomi* zoospore solution, attached to the stem of an *E. marginata* seedling in the glasshouse.

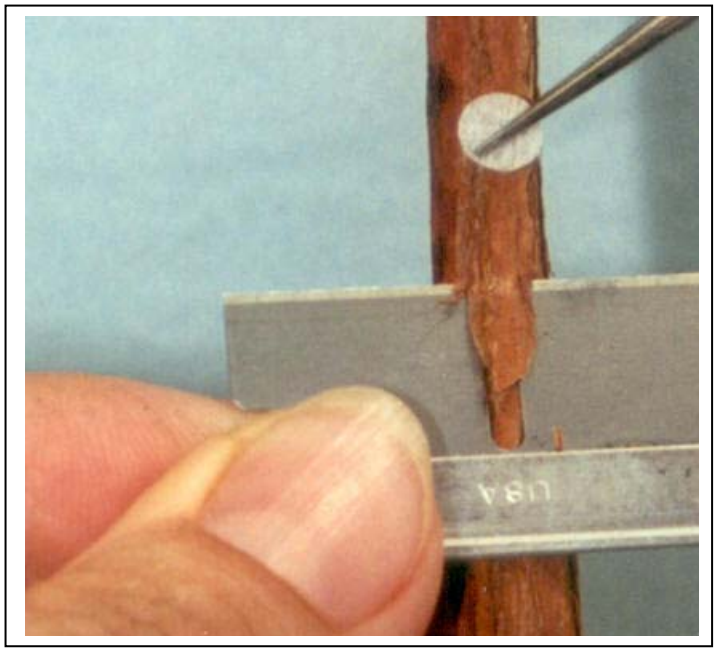


Figure 3.2 Insertion of a Miracloth disc, colonized with *P. cinnamomi* mycelium, into the incision made in the stem of an *E. marginata* seedling.

3.2.7 *Watering regimes*

Three different watering regimes were imposed for both zoospore-inoculated and for underbark-inoculated plants (Table 3.1). All containers with plants were weighed daily and moisture levels kept at the pre-determined level. Briefly, for one third of the seedlings, the substrate was kept at container capacity (CC) throughout the trial by watering to saturation twice daily. Water was withheld from all other containers after inoculation until the seedlings reached wilting point (WP). After WP was established, substrate water content was increased to the pre-determined level (Chapter 2). This level was the weight at which containers were maintained (W_M), which was the weight when 10% of the moisture lost from the weight at container capacity (W_{CC}) to the weight at wilting point (W_{WP}) was restored (Equation 3.2).

$$\text{Equation 3.2:} \quad W_M = W_{WP} + 10\%(W_{CC} - W_{WP})$$

This level was maintained until Harvest 1, 35 days after inoculation, when all surviving underbark-inoculated plants and a proportion of zoospore-inoculated plants were harvested. Half the remaining droughted seedlings, both inoculated and non-inoculated, were maintained at that level until Harvest 2, 63 days after inoculation, while the other half were restored to CC after 14 days of droughted conditions (Fig. 3.3).

3.2.8 *Monitoring for water deficit and plant stress.*

Xylem pressure potential (XPP) of all droughted seedlings was determined at wilting point (WP) using a pressure chamber similar to that described by Scholander *et al*. (1965). In the case of sudden, early death, the WP was taken as the day before death and harvest of the seedling. A comparison was made of the WP between all zoospore-inoculated seedlings and underbark-inoculated seedlings, between zoospore-inoculated and underbark-inoculated seedlings included in Harvest 1 and between zoospore-inoculated seedlings and underbark-inoculated seedlings included in Harvest 2. Length of visible surface lesions and observations on the general health of the plants were recorded each day.

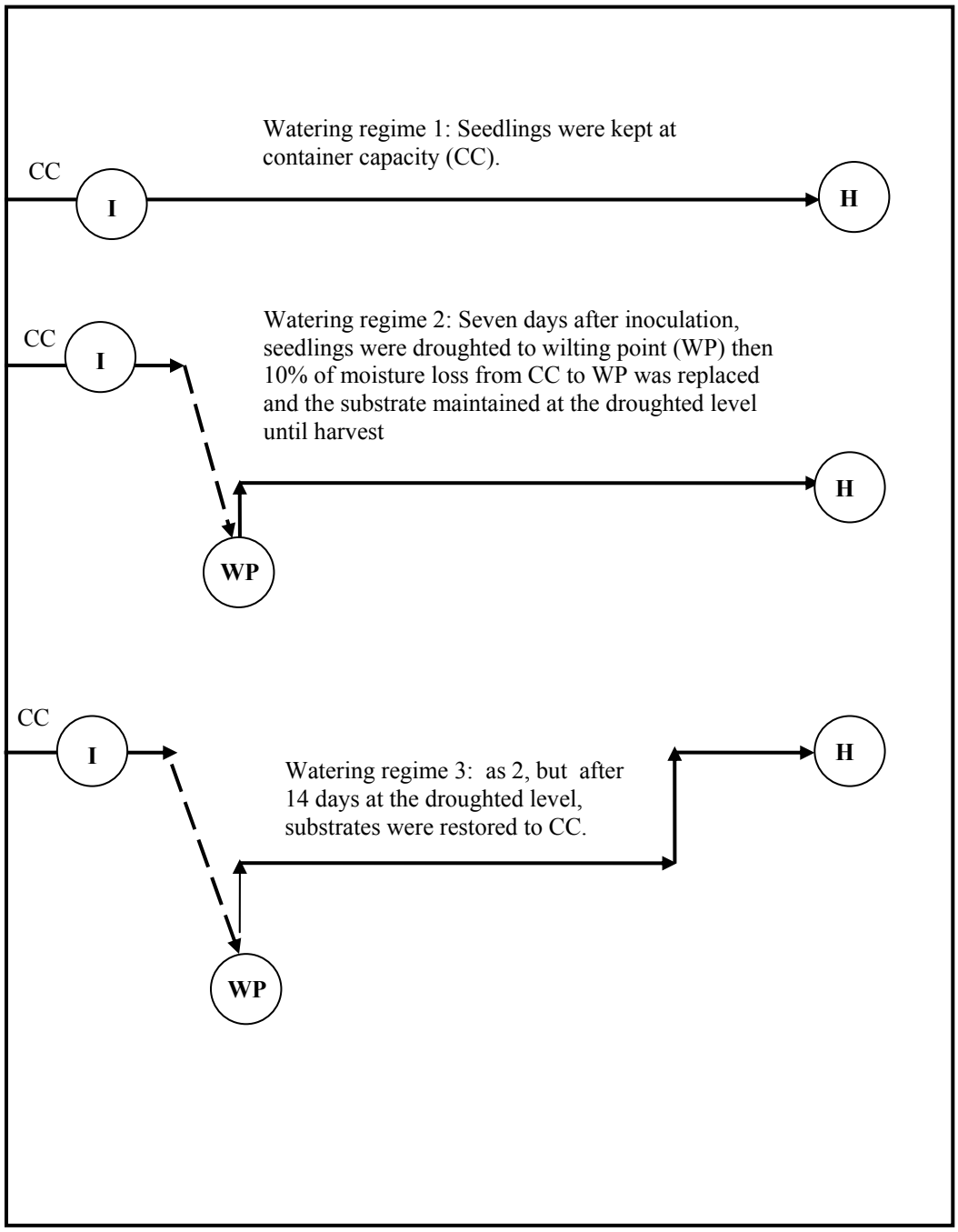


Figure 3.3 Schematic diagram showing the three watering regimes employed, in relation to the time of inoculation (**I**) and final harvest (**H**).

Watering regimes:
(1) substrates of seedlings were kept at container capacity (CC);
(2) 7 days after inoculation, seedlings were droughted to wilting point (WP), then 10% of moisture loss from CC to WP was replaced and the substrate maintained at that level until harvest;
(3) as (2), but after 14 days at the droughted level, the moisture content of the substrate was restored to CC.

3.2.9 *Harvests*

Prior to Harvest 1 (35 days after inoculation), any seedlings from any treatment, which were severely wilted and near to death were harvested. The data collected were added to Harvest 1 results which included all surviving underbark-inoculated seedlings and underbark control plants as well as zoospore-inoculated seedlings not required for the continuation of the experiment. The remaining zoospore-inoculated seedlings and control plants were harvested 63 days after inoculation (Harvest 2) (Table 3.2). At each harvest, visible surface lesions were measured and the stems were cut from 20 cm above the lesion front and down to it in 0.5 cm segments. Some lesions were noted below the ROI and some coppice stems were affected. To standardize the results, only lengths of lesions and colonization above the ROI were included. If no surface lesion was visible, the stem was cut 20 cm above the ROI, then in 0.5 cm segments down to the ROI. The segments were cut longitudinally and plated, cut surface face down, onto NARPH selective agar in 90 mm Petri dishes. These plates were sealed with Parafilm™ and incubated in the dark at 24±1^{o}C. Half of each segment taken at the ROI, at the lesion front (LF) and at 2 cm above the LF was plated as described and the other half was immersed in fixative (Appendix 4) for histological examination. Plates with stem segments were examined daily for evidence of *P. cinnamomi*. The extent of infection of the plant tissue, indicated by the lesion length and/or the infected stem segments above the ROI, and including it, will be referred to as total colonization.

If no *P. cinnamomi* was observed growing from any segment of an inoculated seedling stem after 7 days, the segments were immersed in sterile distilled water in McCartney bottles and rinsed several times over three days. They were then transferred to shallow aluminium foil trays containing soil filtrate (100g soil/litre SDW). Young leaves of *Pimelea ferruginea* were floated on the surface of the filtrate to bait the pathogen. After 7 days they were lifted from the filtrate and excess moisture removed from the leaf surface before they were plated onto NARPH. Since the filtrate had been tested for the absence of *P. cinnamomi* before segments and leaves were introduced, any growth of *P. cinnamomi* from the leaves would indicate that the stem segment had been infected and that the immersion in filtrate had encouraged mycelial growth and sporangial formation.

3.2.10 *Histology*

Portions of stem segments as described (3.2.9), were immersed in a modified version of Karnovsky's fixative (Appendix 4) in eppendorf tubes. Trapped air was removed to ensure better fixation by placing eppendorfs with segments under vacuum for 3 x 5 minute periods, before storage at 4^{o} C. Segments were embedded in Parafin (Paraplast, Oxford Labware, Divison of Sherwood Medical, St Louis, MO 63103, USA) wax, before transverse sections of the ROI and LF, as well as longitudinal sections of the segment 2 cms above the LF were cut with a microtome (Spencer 820, American Optical Corporation, Buffalo N.Y. 14215). The thin sections (10μm thick) were mounted onto glass slides, stained with safranin and Fast green (Jensen, 1962) to detect lignin and were examined with a light microscope (Photomicroscope III, Carl Zeiss, 7082 Oberkochen, Germany). A digital camera (Olympus DP10, Olympus Optical Company Ltd., Shinjuku-ku, Tokyo, Japan) was used to record the images.

3.2.11 *Statistical analysis*

Data were analysed using Statistica® for Windows Version 5 (StatSoft Inc., OK, USA) and Excel for Windows (Microsoft Corporation, USA), Microsoft Excel Analysis ToolPak (GreyMatter International, Inc., Cambridge, MA 02141). Prior to parametric analyses, data were screened for basic assumptions of parametric tests including normal distribution, equality of variances and non-correlation of means and variances. In cases where data did not conform to assumptions, square root or logarithmic (x+1) transformations were used to correct the deviations. The use of such transformations is noted in the relevant results, but means are presented graphically for non-transformed data. After comparison of data between or within plant groups (Table 3.2), where an ANOVA resulted in a P-value of < 0.05, data were considered significantly different.

3.3 Results

3.3.1 *Mortality prior to Harvest 1*

The rate of mortality of underbark-inoculated seedlings was too sudden to allow the implemention of all 3 watering regimes (Fig 3.3). One zoospore-inoculated seedling and 2 underbark-inoculated seedlings died 7 days after inoculation, while all

seedlings were at container capacity. Three days later, 15 seedlings died. The trend continued (Fig. 3.4). By Day 24, 41 of the 48 underbark-inoculated seedlings and 16 of 48 zoospore-inoculated seedlings were dead. At Day 35 the decision was made to harvest all remaining underbark-inoculated seedlings and reallocate the surviving zoospore-inoculated seedlings to complete the experiment based on the original protocol, but with fewer replicates (Table 3.2).

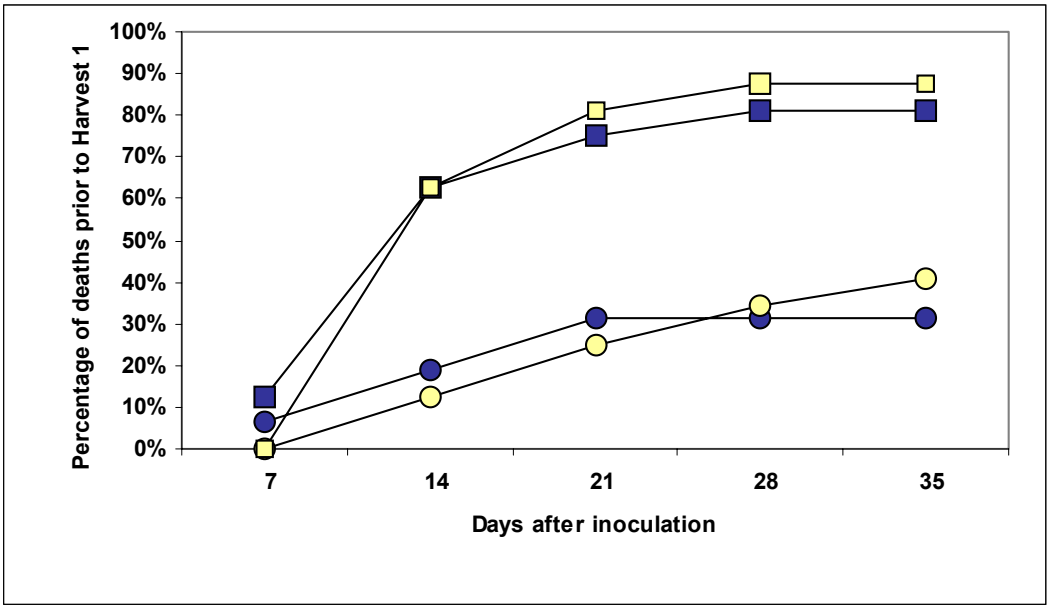


Figure 3.4 Mortality of inoculated *E. marginata* seedlings prior to Harvest 1, 35 days after inoculation under watering regimes 1 and 2 (as described below).

● Zoospore-inoculated seedlings in watering regime 1 were kept at container capacity (CC) throughout.
○ Zoospore-inoculated seedlings in watering regime 2 were droughted to wilting point, then 10% of mositure lost from container capacity to wilting point was restored and the substrate maintained at that level until harvest.
■ Underbark-inoculated seedlings in watering regime 1 were kept at CC throughout.
□ Underbark-inoculated seedlings in watering regime 2, as described above.

Table 3.3 Deaths of *E. marginata* seedlings inoculated with *P. cinnamomi* and subjected to three different watering regimes.

	Deaths prior to Harvest 1				Deaths prior to Harvest 2	
	Underbark		Zoospore		Zoospore	
Watering regime	Inoculated *n* =	Control *n* =	Inoculated *n* =	Control *n* =	Inoculated *n* =	Control *n* =
1	14 (16)	0 (8)	5 (7)	0 (1)	3 (9)	0 (7)
2	28 (32)	0 (16)	13 (14)	2 (2)	1 (9)	0 (7)
3	n/a	n/a	n/a	n/a	0 (9)	0 (7)

Numbers in brackets denote total number of seedlings harvested and include the deaths prior to harvest, shown without brackets. Harvest 1 was 35 days after inoculation and Harvest 2 was 63

days after inoculation. Control plants were sham inoculated with sterile distilled water or with sterile Miracloth™ discs. *n* = number of replicates.
n/a = not applicable. Watering regimes 1, 2 and 3 were as illustrated in Figure 3.3.

3.3.2 *Plant growth*

At the start of the experiment, the mean height of seedlings assigned to zoospore treatments was 65.85±1.49 cm (*n* = 72) and mean height of seedlings assigned to underbark treatments was 60.36±1.47 cm (*n* = 72). A one-way ANOVA gave a significant (*df* 1,142; P=0.01) difference in the heights between inoculation techniques. Heights, at the beginning of the experiment, of zoospore-inoculated seedlings assigned to the 3 different watering regimes prior to Harvest 2, were significantly (*df* 5,42; P <0.001) different. A 2-way ANOVA showed that inoculation status had no significant (P>0.05) effect on relative plant growth but that the watering regime had a significant (*df* 2,39; P=0.016) effect (Fig. 3.5).

There was no significant (*df* 1,142; P >0.052) difference in stem diameters of seedlings, measured at the start of the experiment, between inoculation treatments;. Mean stem diameter for zoospore-inoculated seedlings and control plants was 0.62±0.02 cm and for underbark-inoculated seedlings and controls was 0.60±0.01 cm.

3.3.3 *Wilting point*

Days to wilting point

There was a significant (*df* 1,62; P<0.001) difference in days to WP of non-wounded, zoospore-inoculated seedlings (18.63±0.92 days; *n* = 32) and wounded, underbark-inoculated seedlings (13.38±0.78 days, *n* = 32). In the control plants, there was no significant (P>0.05) difference between non-wounded seedlings, sham-inoculated with SDW (18.13±0.73 days, *n* = 16) and the wounded seedlings, sham-inoculated underbark (20.56±1.37 days, *n* = 16).

Xylem pressure potential at wilting point

Xylem pressure potential at wilting point ranged between –0.6 MPa and –3.5 MPa. One reading of –4.6 MPa from the leaf of a droughted, zoospore-inoculated seedling was omitted because the petiole was damaged when pressure was applied in the chamber. There was no significant (P>0.05) difference in XPP at WP between

underbark-inoculated plants and zoospore–inoculated plants, nor between inoculated and control plants.

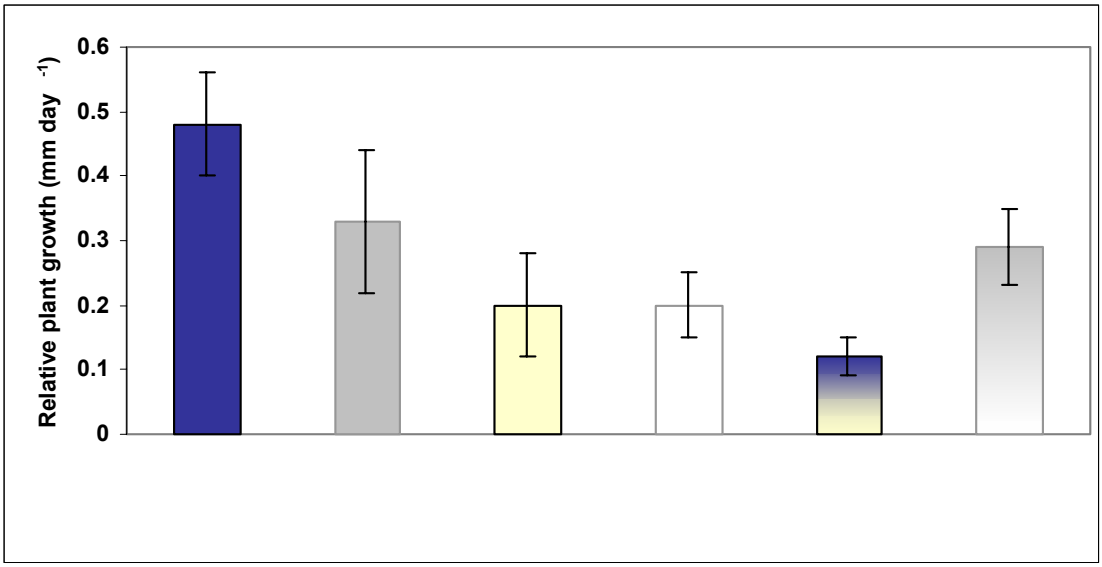


Figure 3.5 Plant growth, relative to Height 1 (Equation 3.1), of zoospore-inoculated *E. marginata* seedlings and corresponding control plants in three watering regimes (see key below) at Harvest 2. Bars represent the standard error of the mean.

Watering regime 1 Inoculated seedlings kept at container capacity (CC) throughout the trial.

Watering regime 1 Control (sham-inoculated) seedlings kept at container capacity (CC).

Watering regime 2 Inoculated seedlings droughted to wilting point (WP) then maintained until harvest at a droughted level, with 10% of substrate moisture lost from CC to WP restored.

Watering regime 2 Control (sham-inoculated) seedlings droughted as in Watering regime 2.

Watering regime 3 Inoculated seedlings droughted as in 2, but restored to CC after 14 days of droughted conditions.

Watering regime 3 Control (sham-inoculated) seedlings droughted then restored to CC as in Watering regime 3.

3.3.4 *Lesions and colonization (Harvest 1)*

Lesions became visible as they grew beyond the stem periderm. Of the 45 stem lesions visible prior to Harvest 1, 35 were seen on underbark-inoculated seedlings and

10 on zoospore-inoculated seedlings. The 3 longest lesions, measured from the lesion front down to the ROI (24, 21 and 15.5 cm) were seen on underbark-inoculated seedlings kept at CC. The greatest extent of colonization by *P. cinnamomi* was also in seedlings in this treatment (Fig. 3.6). Though 4 of the 16 stems displayed no lesions, after segments were plated onto selective agar, *P. cinnamomi* was recovered from all underbark-inoculated seedlings where the substrate was kept at CC. Of the 32 droughted underbark-inoculated seedlings, *P. cinnamomi* was recovered from 28 stems, 5 of which had no lesions. Baiting with *Pimelea ferruginea* leaves did not result in successful recovery of *P. cinnamomi* from the remaining 4 stems. *P. cinnamomi* was recovered from 44 of the 48 underbark-inoculated seedlings (92%) (Table 3.4).

A 2-way ANOVA of square-root transformed data showed that the inoculation technique had a significant (*df* 1,65; P=0.003) effect on total colonization by *P. cinnamomi* in Harvest 1, but that the watering regime did not have a significant (P>0.05) effect. No lesions were observed on any control plants and no *P. cinnamomi* was recovered from any controls in this experiment. There was no correlation between total colonization of stems and the days to WP in any treatment.

Table 3.4 Number of seedlings with lesions and/or colonization at 35 days (Harvest 1) and 63 days (Harvest 2) after inoculation. There were 3 watering regimes, only 2 of which were able to be implemented prior to Harvest 1.

	Harvest 1						Harvest 2		
Watering	Zoospore Inoculated			Underbark inoculated			Zoospore inoculated		
regimes #	1	2	3	1	2	3	1	2	3
Lesions	2	8	n/a	12	23	n/a	3	5	4
Colonization	5	12	n/a	16	28	n/a	5	6	5
Nil recovery	2	2	n/a	0	4	n/a	4	3	4
Totals	7	14		16	32		9	9	9

Lesions = visible lesions only. Colonization includes all infected plants with and without visible lesions. Colonization + Nil recovery = Total number of plants in each treatment. n/a = not applicable.
Watering regimes 1, 2 and 3 were as illustrated in Figure 3.3.

3.3.5 *Mortality prior to Harvest 2*

Four deaths occurred before the final harvest of zoospore-inoculated seedlings. Three were seedlings that had been kept at CC throughout the trial, the fourth was a

seedling maintained at the droughted level. These four were harvested at death, 47 and 48 days after inoculation; all other seedlings were harvested at Day 63.

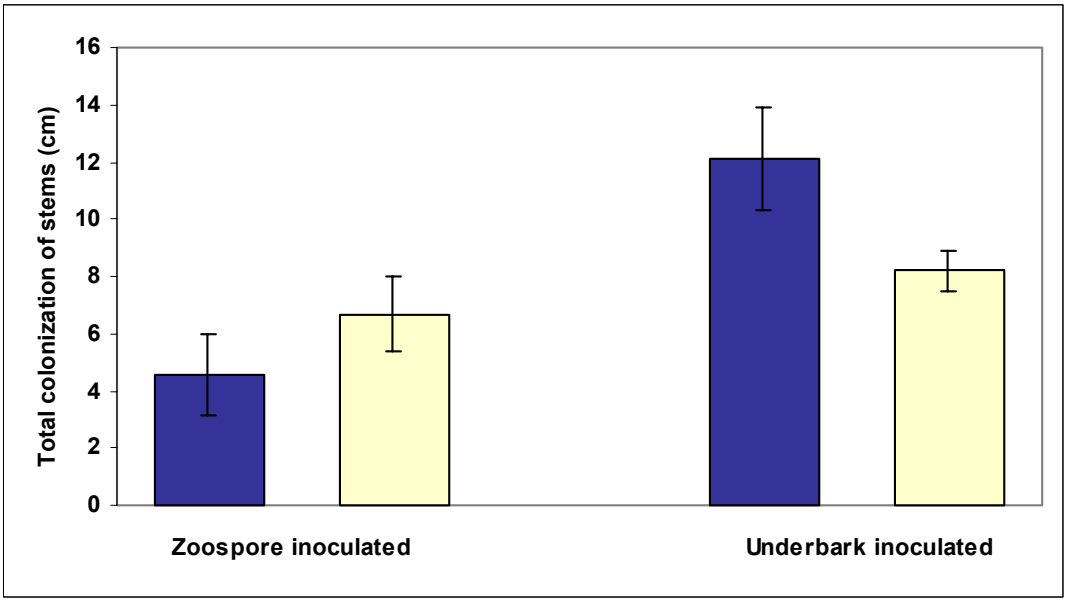


Figure 3.6 Extent of colonization of *E. marginata* seedlings at death or at Harvest 1, 35 days after inoculation. Total colonization is the length of lesions plus the colonization by *P. cinnamomi* of plant tissue beyond the lesion front.
Bars represent the standard error of the mean.

Watering regime 1 Inoculated seedlings kept at container capacity (CC) throughout the trial.

Watering regime 2 Inoculated seedlings droughted to wilting point (WP) then maintained until harvest at a droughted level, with 10% of substrate moisture lost from CC to WP restored.

3.3.6 *Lesions and colonization* (Harvest 2)

All Harvest 2 seedlings were zoospore-inoculated; none were underbark-inoculated. At harvest, 63 days after inoculation, the longest visible surface lesion of 28.5 cms was on a seedling which had been kept at CC throughout the experiment. Two seedlings kept at CC, which died prior to harvest, had lesions of 18.5 cms. One seedling, which had been droughted and restored to CC had a lesion of 23 cms but survived until harvest. The stem of another seedling, which died 47 days after inoculation, was severely constricted at the ROI but no lesion was discernible. No colonization was found beyond the 1 cm ROI in this seedling. An ANOVA of log-

transformed data showed no significant (P>0.05) difference in colonization between the 3 watering regimes (Fig. 3.7).

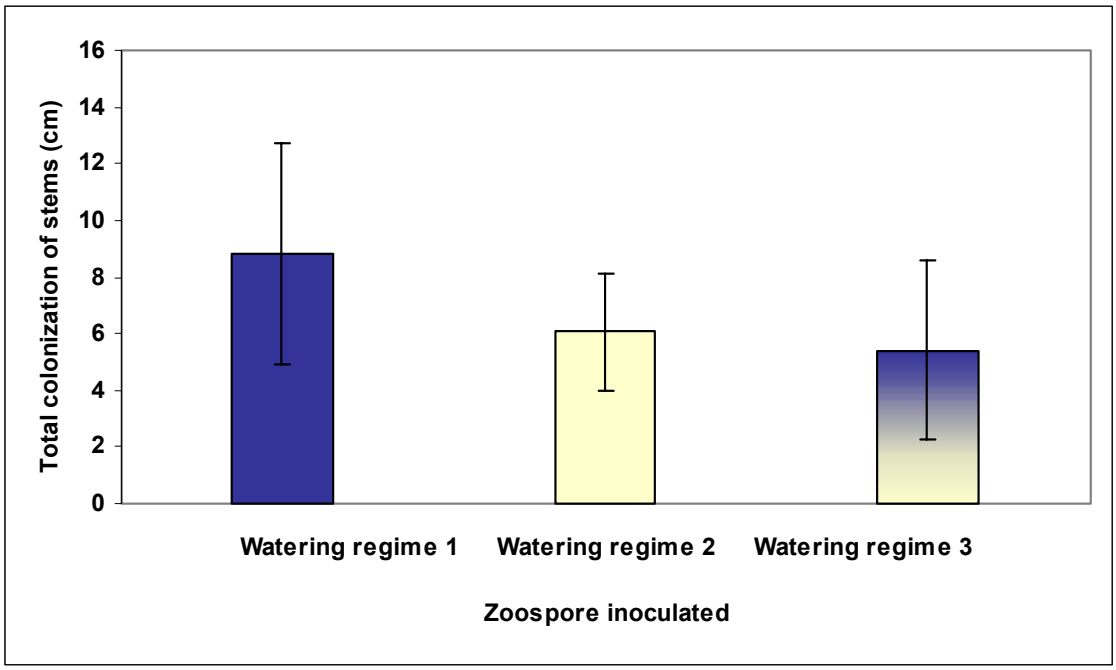


Figure 3.7 Extent of colonization in zoospore-inoculated *E. marginata* seedlings in three different watering regimes, 63 days after inoculation (Harvest 2). Total colonization is the length of lesions plus colonization of plant tissue beyond the lesion front. Bars represent the standard error of the mean.

Watering regime 1 Inoculated seedlings kept at container capacity (CC) throughout the trial.

Watering regime 2 Inoculated seedlings droughted to wilting point (WP) then maintained until harvest at a droughted level, with 10% of substrate moisture lost from CC to WP restored.

Watering regime 3 Inoculated seedlings droughted as in 2, but restored to CC after 14 days of droughted conditions.

3.3.7 *Recovery after Harvest 2 and histology*

P. cinnamomi was recovered from the stem segments of 16 of the 27 inoculated seedlings in the second harvest, irrespective of treatment. There were no lesions on the 11 stems from which *P. cinnamomi* was not recovered when directly plated onto NARPH agar. No further recoveries were made after baiting. Over the 2 harvests, *P. cinnamomi* was recovered from the stems of fewer zoospore-inoculated plants (33) than underbark-inoculated plants (44), 69% and 92%, respectively. After histological

preparation, microscopy revealed evidence of defence barriers (lignification) in healthy tissue adjacent to the diseased cells in stems (Figs. 3.8 and 3.9).

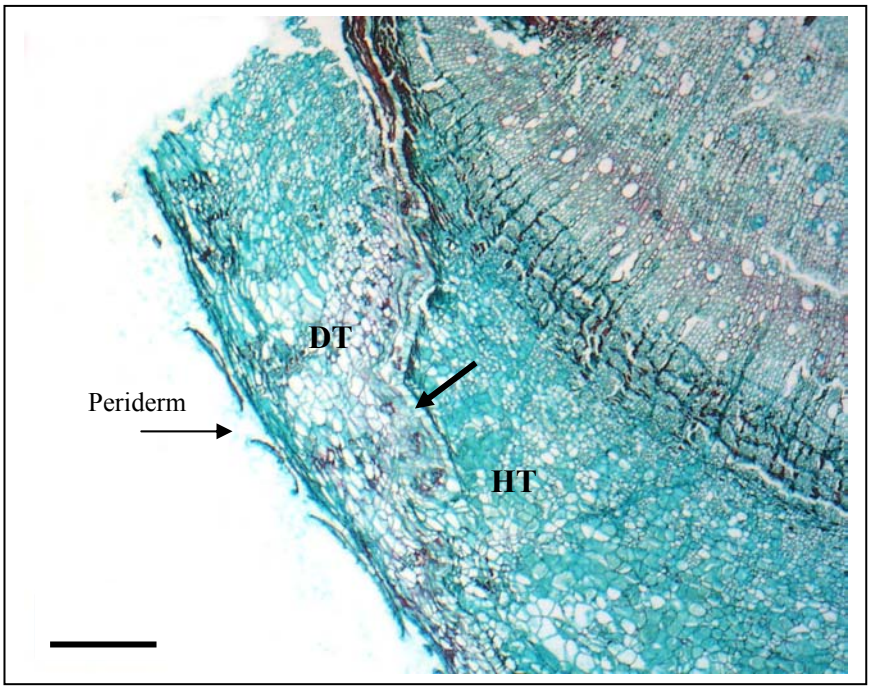


Figure 3.8 Transverse section of a droughted *E. marginata* seedling stem inoculated with *P. cinnamomi* zoospores. The section was taken from the region of inoculation at Harvest 2 (63 days after inoculation) and stained with safranin and Fast green. Lignification (thick arrow) had formed a barrier between the healthy tissue (HT) and the diseased tissue (DT). Loss of integrity can be seen in the diseased cells. Scale bar =200 µm

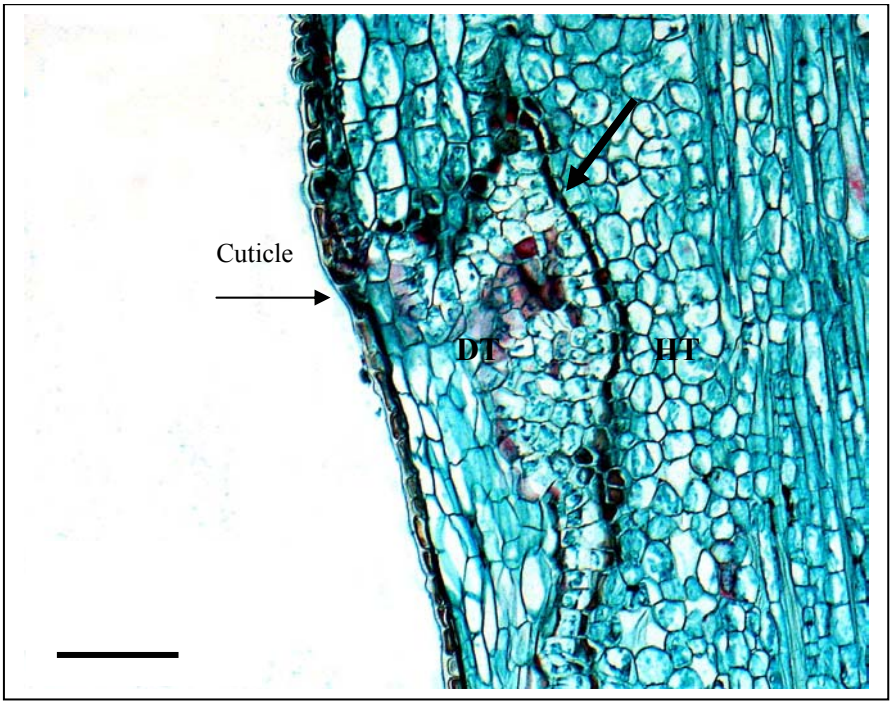


Figure 3.9 Longitudinal section of the same *E. marginata* stem, taken 2 cm beyond the 17.5 cm visible lesion front and stained with safranin and Fast green. Lignification (thick arrow) formed a barrier between the healthy tissue (HT) and the diseased tissue (DT). Scale bar = 100 µm.

3.4 Discussion

3.4.1 *Comparison of inoculation techniques*

There was a marked difference in the infection of *E. marginata* stems by *P. cinnamomi* between the two inoculation techniques. The greatest extent of colonization at Harvest 1, the most recoveries of *P. cinnamomi* and the highest rate of mortality were in underbark-inoculated seedlings. The lesser extent of colonization in zoospore-inoculated seedlings compared to underbark-inoculated seedlings and the fewer recoveries in zoospore-inoculated seedlings compared to underbark-inoculated seedlings agrees with the findings of O'Gara (1998), who used, concurrently, similar inoculation methods on *E. marginata* stems. Cahill *et al*. (1989) found a difference in the response of cortical cells of *E. marginata* roots to inoculation with zoospores and inoculation with mycelium of *P. cinnamomi*. However, that study examined the inoculation, at lower temperatures, of roots of two-month old seedlings while the current study is of 12-month old stems.

Introduction of *P. cinnamomi* immediately after wounding gives the seedling less time to implement biochemical and physical defence strategies than when infection occurs without wounding. An injury to healthy plant tissue can invoke physiological responses, at the boundary between damaged and unaffected tissue, in membranes, cytosol and cell walls as precursors to the healing process (Bostock and Stermer, 1989). Wounding of the stem of the host plant, immediately followed by direct introduction of *P. cinnamomi* mycelium to the phloem sugars, facilitates the establishment of the pathogen in plant tissue and disadvantages the host. In contrast, interaction between host and pathogen after inoculation of a non-wounded stem with zoospores of *P. cinnamomi*, would allow recognition by the host of the pathogen and allow more time for defence strategies to be effected. The level of total phenolics *in planta*, particularly in C. *calophylla,* which were resistant to *P. cinnamomi* (Cahill and McComb, 1992) or *E. marginata* clonal plants resistant to *P. cinnamomi*, increases after inoculation with the pathogen (Cahill *et al*., 1993; Burgess *et al*., 1999a). Lignification of the middle lamella and subsequently the cell walls can be induced following the *de novo* synthesis

of phenylalanine ammonia lyase (PAL) and peroxidase (Vance *et al*., 1980). This structural defence can retard the incursion of a pathogen but may not always be an effective barrier (Vance *et al*., 1980; Cahill *et al*., 1989).

The impact that the underbark inoculation method had on seedlings compared to the non-wounding method was clearly shown. The percentage of underbark-inoculated seedlings infected (92%) was much greater than zoospore-inoculated seedlings (69%), but the rapid mortality rate that resulted from the former technique was a disadvantage for prolonged observation of disease development under different watering regimes. Although the percentage of underbark-inoculated seedlings infected by *P. cinnamomi* was much greater than that of zoospore-inoculated seedlings, this trial demonstrated that wounding is not necessary for infection to occur in stems of 12 month old *E. marginata* seedlings and confirms the findings of O'Gara *et al*. (1996).

3.4.2 *The role of watering regimes in disease development*

Watering regimes played a major role in disease development and expression, illustrating that moisture content of the substrate, and consequently of the host plant, was an important factor that facilitated or impeded progress of *P. cinnamomi*. This was particularly evident in underbark-inoculated seedlings, where the amount of colonization found in seedlings kept at container capacity was significantly greater than that of droughted seedlings at Harvest 1. This finding was supported with colonization data of zoospore-inoculated seedlings in Harvest 2, though the difference in colonization of stems between the three watering regimes was not statistically significant. Harvest 1 data for colonization of zoospore-inoculated seedlings did not support the finding. However, the lower replicate numbers (n = 7) for zoospore-inoculated seedlings kept at CC in Harvest 1, necessary to allow the continuation of the experiment, may have biased the results. It should also be considered that seedlings for which the substrate was restored to container capacity prior to Harvest 2, would have been droughted for a longer period of time than those selected for the first harvest. This may have given droughted seedlings in Harvest 2 a greater chance to contain the *P. cinnamomi* compared to seedlings kept at container capacity throughout the trial. This supports the findings of Bunny *et al*. (1995) who found increased susceptibility and longer lesions in inoculated *E. marginata* trees at field sites with higher rainfall than at sites where trees were water-stressed. In the current glasshouse study, the higher

moisture content in tissue of seedlings kept at container capacity would advantage *P. cinnamomi* which requires water for vegetative reproduction and for the infection process *in planta.* This would explain the extent of colonization in zoospore-inoculated seedlings at Harvest 2 (Fig. 3.7).

No seedlings were droughted prior to inoculation in this trial. As they were kept at CC for 7 days after inoculation, the pathogen was able to become well established in plant tissue before stem moisture was diminished. The timing of the inoculation, 7 days prior to water being withheld in droughted treatments, has implications for the results and, coupled with fewer replicates, may explain the lower mean total colonization of the zoospore-inoculated seedlings in Harvest 1 (Fig. 3.6). Imposing water deficit on seedlings immediately after or prior to inoculation would mean lower stem moisture and this aspect is examined in later experiments (Chapters 6, 7 and 8).

Results for Harvest 2 show that in the zoospore-inoculated seedlings, the mean total colonization of plants kept at container capacity (CC) was higher than that of plants subjected to either of the droughted treatments. Although the difference was not statistically significant, these results indicate that high substrate moisture and consequent high plant tissue moisture is a crucial factor for the pathogen to become established in the phloem or the xylem of the host. Tippett *et al*. (1987) found that *P. cinnamomi* was less inhibited in *E. marginata* seedlings with reduced water deficits than in water-stressed seedlings.

3.4.3 *Other factors affecting disease development*

Seedlings were monitored daily to determine wilting point, defined by loss of turgidity in tip growth. This observation was rather subjective. Since the weight of the container, with substrate and plant, on the day that wilting point was reached, was used to calculate the level of water deficit that would be imposed on each replicate (Equation 3.2), any inaccuracy in the nomination of the day of wilting point would result in variation in the actual level of stress imposed on the droughted seedlings. This could explain the deaths, prior to Harvest 1, of two droughted control plants and two droughted, underbark-inoculated seedlings from which *P. cinnamomi* was not recovered. The level of water stress that was applied was determined by the droughting trial described in Chapter 2. The nine-month-old *E. marginata* seedlings in that droughting trial were healthy, smaller plants, not inoculated and able to adjust

osmotically as drought was imposed step-wise. They survived when maintained with a restoration of 5% of moisture lost from container capacity to final wilting point. In the current experiment, the allocation of an immediate droughted level of 10% restoration after wilting point may not have been adequate to compensate for the increased transpiration rate in all the older, taller seedlings, half of which were inoculated with the pathogen and they may not have been able to adjust as well osmotically to the lower percentage of restoration of moisture lost from CC to WP (10% *cf* initial restoration of 30% in Chapter 2).

The xylem pressure potential (XPP) readings gave no indication of different levels of plant stress at wilting point either between the two inoculation techniques, or between inoculated and control plants. While the determination of wilting point was subjective, it would be expected that droughted, diseased plants would register a greater level of stress (i.e. more negative readings (MPa) on the gauge of the pressure chamber) than droughted, healthy plants. After inoculating *Xanthorrhoea preissii*, an understorey species in the *E. marginata* forest, with *P. cinnamomi*, Pilbeam *et al*. (2000) found no significant (P>0.05) difference in XPP between inoculated and non-inoculated plants in droughted conditions. Stoneman *et al*., (1994) in a greenhouse study of *E. marginata* seedlings found no significant (P>0.05) difference in XPP between droughted plants and well watered controls and Colquhoun *et al*., (1984) found little seasonal variation in XPP in *E. marginata* trees. Crombie and Milburn (1988) in a paper that mentions "dieback" but not *P. cinnamomi*, found no significant (P>0.05) difference in the midday XPP of dieback affected plants and controls of other *Eucalyptus* spp. Wilting point was the constant for all readings in the current experiment and precautions for possible error were taken (Ritchie and Hinckley, 1975). However, the widely used method of quantifying plant stress levels by recording xylem pressure potential using the pressure chamber (Scholander *et al*., 1965), may not always be the most accurate technique for detecting variations in stress levels of some species of plants in the glasshouse. Other methods (e.g. stomatal conductance) will be considered in future experiments.

Temperatures in the glasshouse during this experiment were close to ideal for the pathogen. Hüberli *et al*. (1997) found that growth of the same *P. cinnamomi* isolate used in the current experiment increased in temperatures between 16-28^{o}C and declined in temperatures above 28^{o}C. The experiment was conducted in winter months when temperatures in the glasshouse ranged from 12.5^{o}C to 26.9^{o}C apart from one day where

the maximum was 30.5°C. This temperature range, coupled with the immediate introduction of an aggressive isolate into a deliberate phloem-deep wound, would contribute to the high impact that *P. cinnamomi* had on the underbark-inoculated seedlings. At the time of inoculation, the temperature of water in the inoculation receptacles attached to the stems, was 21 to 22°C.

The differences in lesion lengths and in colonization by *P. cinnamomi* reflect the variations in expression of the disease and disease development *in E. marginata* seedlings. They also illustrate the contrasting outcomes that may result from different inoculation techniques. In zoospore-inoculated seedlings kept at container capacity, the extremes of disease expression were manifested in (i) a seedling which died 7 days after inoculation, (ii) a severely constricted region of inoculation, beyond which the pathogen was not recovered, on a seedling which died 47 days after inoculation and (iii) visible surface lesions up to 28.5 cms on seedlings which survived until Harvest 2, 63 days after inoculation. The broader range of genetic material in seedlings than in clonal plants (Chapters 5, 6 and 7) may have contributed to the variance in the expression of the disease. It is clear that the genotypic as well as physiological variations between seedlings contributed to the level of disease development.

Recovery of *P. cinnamomi* from inoculated stems was not always successful, even after leaching with sterile distilled water and the baiting with *Pimelea ferruginea* leaves while stem segments were immersed in soil filtrate. Techniques for improved recovery were investigated in later work (Chapter 9). Failure to recover *P. cinnamomi* from some zoospore-inoculated stems may indicate that the inoculation of these stems was not successful, that the recovery technique needed to be improved or that the possibly hypersensitive response of the more resistant seedlings prevented colonization by the pathogen.

That substrate moisture level is crucial to the development of disease caused by *P. cinnamomi* in *E. marginata* seedlings, using either inoculation technique, is strongly indicated by the results obtained in this trial. This factor and the timing of inoculation will be re-examined in later experiments (Chapters 6, 7 and 8).

3.4.4 Conclusion

Neither inoculation technique was considered suitable for application in the field trial (Chapter 5). Firstly, the underbark inoculation technique was too invasive and resulted in rapid death of seedlings so that disease could not be monitored over an extended period of time. Secondly, the zoospore inoculation method resulted in fewer deaths but had the disadvantages of (1) the percentage of seedlings which became infected was not as high as with the underbark method with droughted seedlings and (2) it presented logistical challenges in the field. Receptacles would have to be attached to several hundred stems and half that number would be filled with zoospore suspension as inoculum. There would be no certainty of successful production of a high concentration of zoospores in sufficient volume in the field in the required time. Further research (Chapter 4) developed a new inoculation technique better suited to the criteria set by the design of the major field trial (Chapter 5).

Chapter 4

A new, rapid and non-invasive technique to inoculate plants with *Phytophthora cinnamomi*

The content of the chapter was published as:
Lucas, A., Colquhoun, I.J., McComb, J.A. and Hardy, G.E.St.J. Hardy (2002) – A new, rapid and non-invasive technique to inoculate plants with *Phytophthora cinnamomi*. *Australasian Plant Pathology* **31**: 27-30. A. Lucas conducted the research and the other three authors were supervisors of the PhD project.

4.1 Introduction

The ability of *Phytophthora cinnamomi* to infect root tips and lateral roots of susceptible plant species via motile zoospores has been well documented (Zentmyer 1980; Erwin and Ribeiro 1996). This pathogen is an impediment to the successful rehabilitation of bauxite mines in the jarrah forest where thousands of hectares are infested by the pathogen (Colquhoun and Hardy 2000). Surface water ponding in rehabilitated mine sites has been found to provide conditions favouring infection through the bark at plant collars of *Eucalyptus marginata* Donn ex Sm. (jarrah), (Hardy *et al*. 1996; O'Gara *et al*. 1996*)*. To study the effect of environmental factors on the expression of the disease in infected plants it is necessary to have a quick, inexpensive and reliable method of inoculating large numbers of plants. There were four criteria to be addressed when developing this technique. A method was required that did not necessitate the wounding of plant tissue; that simulated surface water ponding, often seen in riplines on rehabilitated mine sites; that was applicable to both glasshouse trials and field trials and that was less labour intensive than previously published methods.

A range of inoculation techniques has been reported for the inoculation of plants in the field. Most methods require wounding of the host and include drilling into host tissue and inserting plugs of mycelial agar (Tippett *et al*., 1983), incising the bark and inoculation with colonized Miracloth™ discs (Davison *et al*., 1994; Pilbeam *et al*., 2000) or a colonized mycelial plug (Marks *et al*. 1981; Shearer *et al*., 1987; Bunny *et al.,* 1995). It has been shown that infection of stems of jarrah seedlings or the collars of saplings can occur under field conditions (O'Gara *et al*., 1997). Receptacles constructed around the stems of seedlings were first filled with water to simulate the effect of ponding on the stem then with a solution of zoospores of *P. cinnamomi.* Although their

method avoids wounding and simulates the effect of ponding around the stem, construction of waterproof receptacles is labour-intensive and successful production of zoospores in the field is difficult.

The aim of this experiment was to develop a less labour-intensive, non-wounding method of inoculation which simulated surface water ponding and which could be applied to glasshouse trials and to field trials on rehabilitated mine sites, to enable subsequent studies of the effects of environmental factors on disease development in large numbers of plants infected under controlled conditions.

4.2 Methods

4.2.1 *Experimental design*

This was a multivariate design with four treatments, A, B, C and D (Table 4.1), each with 6 replicate plants. Each plant was inoculated with an agar plug colonized by *P. cinnamomi*, without wounding, on both the periderm and on the green stem tissue. Control plants were sham inoculated with a sterile agar plug. The stems of seedlings in two of the treatments and their controls were pre-treated with wet cotton wool for 2 days prior to inoculation (Table 4.1). At inoculation, agar plugs were held in place on the stems with Parafilm. On half the stems, wet cotton wool was placed over the plug before Parafilm was wrapped around (Table 4.1). All plants were harvested 14 days after inoculation.

Table 4.1 Protocol for inoculation of *Eucalyptus marginata* seedlings with *Phytophthora cinnamomi*

	pre-Treatment of stem*	Inoculation	Moist cotton wool applied at Inoculation	Number of Replicates
A	-	Mycelial agar plug	-	6
	-	Sham agar plug	-	2
B	+	Mycelial agar plug	-	6
	+	Sham agar plug	-	2
C	-	Mycelial agar plug	+	6
	-	Sham agar plug	+	2
D	+	Mycelial agar plug	+	6
	+	Sham agar plug	+	2

* see Section 4.2.3

4.2.2 *Plant material and growing conditions*

Seedlings of *E. marginata* were supplied by Marrinup Nursery (Alcoa World Alumina, Australia) and grown in a shadehouse in 150 mm free-draining containers of peat/perlite [2:1, v/v] with added nutrients (Appendix 1). Eighteen-month-old plants were transferred to a glasshouse, and watered to container capacity daily for 7 days prior to inoculation and throughout the trial. Plants ranged in height from 61 to 86 cm and stem diameters, measured 5 cm above the soil line, were from 0.74 to 0.90 cm. A naturally formed periderm extended at least 10 cm from the base of all stems. The trial was conducted in spring when temperatures in the glasshouse ranged from 13.4^{o}C to 31.9^{o}C.

4.2.3 *Inoculation with* P. cinnamomi

A virulent isolate of *P. cinnamomi*, MU 94-48 (Hüberli, 1995) was grown for a week prior to inoculation on V8 agar (Appendix 2a). Inoculation plugs, 1 cm x 1 cm, were cut from this mycelial agar and sham plugs used on the controls were cut from plates of sterile V8 agar. Balls of cotton wool were autoclaved in deionized water at 121^{o}C for 20 minutes on three consecutive days. Two days prior to inoculation, plants selected for pre-treatment had the sterile, wet cotton wool placed on an internodal area of periderm tissue (about 5-8 cm above the soil) and on the green stem (about 25-30 cm above the point of inoculation on the periderm). These were the intended regions of inoculation. The cotton wool was allowed to retain as much water as possible and was kept in place using Parafilm. It was removed immediately prior to inoculation.

After the 2 days of pre-treatment, all plants were inoculated, or sham inoculated. Inoculum plugs were placed, top-surface against the stem, directly onto the previously moistened areas or corresponding internodal areas on dry stems. In two treatments inoculum plugs were held in place with wet cotton wool and bound into place with Parafilm. In the remaining two treatments only Parafilm was used (Table 4.1). The region of stem in direct contact with the inoculum plug is referred to as the region of inoculation (ROI). Visible lesions were monitored daily for growth.

4.2.4 *Harvest*

Fourteen days after inoculation all plants were harvested and the extent of colonization was assessed. If no surface lesion was visible, 1 cm segments of stem were cut starting 9 cm above the ROI, which was also cut as a 1 cm segment. If surface lesions had developed, 1 cm segments were cut from 9 cm above the lesion front back to the ROI. The 1 cm segments from each stem were cut longitudinally and plated onto NARPH agar, selective for *Phytophthora* (Hüberli *et al*., 2000). The ROI segments were surface sterilized prior to plating onto agar by dipping into 70% ethanol and flaming. The remaining portion of all stems was labelled and kept at 5^{o} C. If *P. cinnamomi* was recovered from segment 9 in the initial plating, the process was repeated with the next 10 cms plated onto selective agar to determine the full extent of colonization in the infected stem.

4.2.5 *Statistical analysis*

Data were analysed using Statistica® for Windows Version 5 (StatSoft Inc., OK, USA) and Excel for Windows (Microsoft Corporation, USA), Microsoft Excel Analysis ToolPak (GreyMatter International, Inc., Cambridge, MA 02141). Prior to parametric analyses, data were screened for basic assumptions of parametric tests including normal distribution, equality of variances and non-correlation of means and variances. After comparison of data between or within treatments (Table 4.1), where an ANOVA resulted in a P-value of < 0.05, data were considered significantly different

4.3 Results

4.3.1 *Recovery of* P. cinnamomi

Results of the plating onto NARPH after harvest showed that *P. cinnamomi* colonized tissue extending beyond that of the surface lesion front in all four treatments, in 21 of the 24 stems inoculated on the periderm region and from all 24 of the inoculated green stems. There was 100% infection in stems that were pre-treated and which also had wet cotton wool applied at inoculation (Treatment D), (Table 4.1). No deaths were recorded in any treatment and no *P. cinnamomi* was recovered from any control plant.

4.3.2 *Lesions and colonization*

Surface lesions had developed by day 7 on green stems of 3 of the 12 inoculated seedlings which had been pre-treated with wet cotton wool before inoculation and in one seedling which had no pre-treatment but the ROI of which had been wrapped in wet cotton wool at inoculation. No lesions appeared on the dry stems. When plants were harvested on day 14, two or more seedlings in all four treatments had developed surface lesions on the green stem. A periderm lesion was observed in only one plant (pre-treated with wet cotton wool and with wet cotton wool applied at time of inoculation). No lesions were observed in either the periderm or the green stem of any control plant. Overall, fewer lesions were observed on stems not given the pre-treatment and those in which the agar plug was not kept moist with wet cotton wool after inoculation (Table 4.1). A one-way ANOVA of total colonization (green stem and periderm combined data) showed no significant (*df* 3,44; P>0.05) difference between treatments. There was no significant (*df* 3,20; P>0.05) difference in colonization of periderm inoculated stems between treatments (Fig. 4.1).

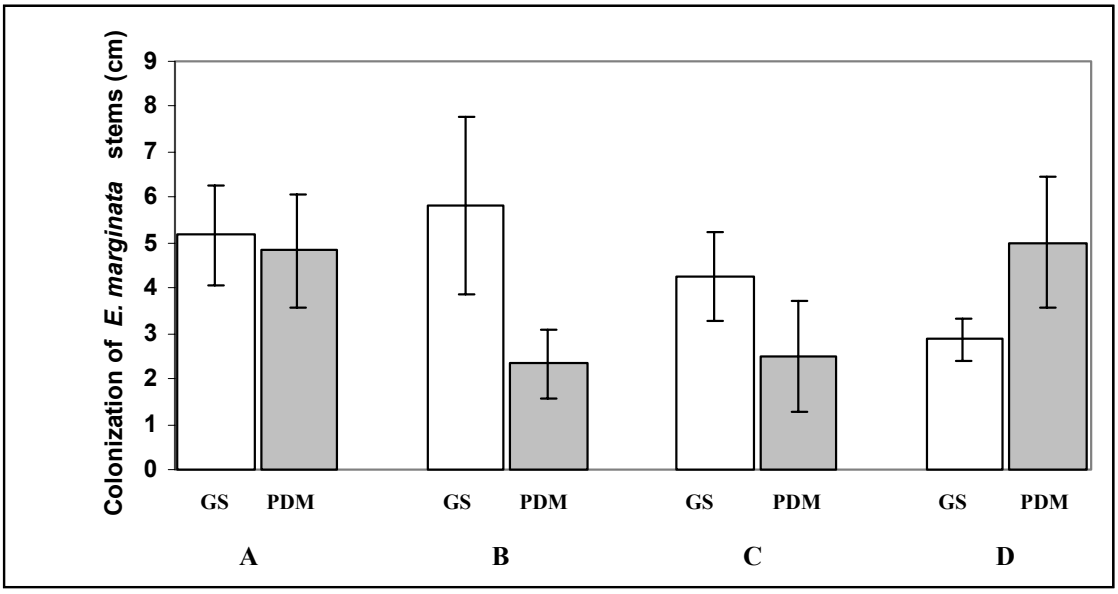


Figure 4.1 Colonization by *P. cinnamomi* above the region of inoculation (ROI) of green stems (left) and periderm (right) of *E. marginata* seedlings in each of the four treatments, A, B, C and D (Table 4.1). GS = Green stem; PDM = Periderm.
Bars indicate standard error of the mean.

4.4 Discussion

It was shown that it is possible to inoculate stems of *E. marginata* plants efficiently without wounding and using mycelium rather than zoospores. The most successful infection of stems with periderm involved pre-treatment of inoculation sites with wet cotton wool for 2 days, then, when the mycelial agar inoculum plug was in place, keeping it moist by the application of a new, wet cotton wool ball, before wrapping. All criteria set prior to development of the technique were met. The technique was simple and rapid to set up, no wounding of the host plant was necessary and stems were successfully inoculated, achieving the required result for application in subsequent experiments. The wet cotton wool provided a faster means of simulating the effect of surface water ponding than the water-filled receptacles used by O'Gara *et al.* (1996). Glasshouse results do not necessarily predict field results, but the high success rate in the glasshouse trial suggested that this inoculation technique could be used in the field. The author and other researchers have since had success using Treatment D on other plant hosts and with other plant pathogens, both in the glasshouse and in the field. In the field an additional wrap of silver ducting tape was used around the ROI to prevent desiccation of the inocula. Treatment D has been used to inoculate clones of *E. marginata* (Chapters 5, 6 and 7*),* selected as resistant or susceptible to *P. cinnamomi* (McComb *et al*., 1994) as well as several other species, including *E. globulus* with *Endothiella* (T. Jackson, *pers. comm*.) and *Grevillea* spp. with *P. cinnamomi* (Dr. M. Dobrowolski, *pers. comm*.). This treatment was selected because it resulted in 100% infection of stems and because it met the criteria set for experimental conditions. Other advantages of the use of wet cotton wool were noted. Agar plugs in treatments without cotton wool tended to break up when Parafilm was wrapped around them; those with the wet cotton wool support did not. At harvest, when the Parafilm was removed from the stem in treatments without cotton wool, the agar plug was partially embedded in the bark and had to be removed carefully prior to surface sterilization of the ROI. This difficulty was not encountered in treatments where the wet cotton wool over the mycelial agar kept the plug intact and allowed it to be easily removed.

Zentmyer (1960) attempted a similar inoculation technique using macadamia seedlings. Potato-dextrose agar, colonized by *P. cinnamomi*, was applied to the unwounded stems of macadamia seedlings 10 to 14 inches in height. The agar was covered with moist cotton and wrapped with adhesive tape. This treatment resulted in

no lesions on the macadamia stems after 4 weeks. Zentmyer concluded that *P. cinnamomi* would only infect macadamia stems after wounding but it is not reported if the pathogen was found to be present after plating the symptomless tissue onto agar.

Histological investigation of stem sections, using a scanning electron microscope (SEM), indicated that colonization was by hyphal penetration. Though stem segments were festooned with hyphae, no sporangia and no zoospores of *P. cinnamomi* were observed either in the tissue of the stem segments or in the cotton wool removed from the ROI at harvest. SEM images showed that the apertures of stomata on *E. marginata* green stems were greater than the diameter of *P. cinnamomi* hyphae, suggesting a potential infection site, but no evidence of hyphal entry via the stomata was observed. This inoculation technique has been proved to be highly effective, and is particularly useful when researchers wish to avoid wounding of host plants or to do extensive inoculation in remote field areas.

Chapter 5

Are drought-stressed *Eucalyptus marginata* plants less susceptible to infection by *Phytophthora cinnamomi* than non-stressed plants? A field study.

5.1 Introduction

Successful revegetation of mined areas of *Eucalyptus marginata* (jarrah) forest can be hampered by the presence of *Phytophthora cinnamomi* in the soil (Colquhoun and Petersen, 1994). The continuing improvement of management strategies and standards of hygiene to combat this pathogen when rehabilitating bauxite mine sites has been well documented (Nichols *et al*., 1985; Bartle and Slessar, 1989; Ward *et al*., 1993; Colquhoun and Hardy, 2000). These improvements, together with the development of clonal lines of *E. marginata* resistant to *P. cinnamomi* (McComb *et al*., 1990; Cahill *et al*., 1992; Stukely and Crane, 1994) and an awareness of seasonal factors contributing to establishment of plant species (Ward *et al*., 1996) and to the development of the disease caused by *P. cinnamomi* in mine sites (Hardy *et al*., 1996; O'Gara, 1998), have resulted in a well planned rehabilitation programme.

Drought has played a major role in the evolution of plant species and ecosystems (Hill, 1994; Wardell-John *et al*., 1997; Scheiner and Rey-Beneyas 1994). In the south-west of Western Australia, the flora has adapted to a mediterranean climate with cool wet winters and long, hot, dry summers, but not to the relatively recent introduction of *P. cinnamomi* within the last two hundred years. In the current study, the interactions between *E. marginata* and *P. cinnamomi* and drought are examined. Although much research into the interactions between *E. marginata* and *P. cinnamomi* has focused on the forest ecosystem, there is little research on these interactions in rehabilitated bauxite mines. Initially, after rehabilitation of the mine pits, the sites are uniform and exposed to different environmental conditions from those of adjacent forest. In the rehabilitated, exposed mine pits (Fig. 5.1), different environmental conditions result from the lack of canopy cover (McChesney *et al*., 1995). With less protection from direct sun and from wind, surface soil moisture is lower, surface soil temperature and ambient air temperature are higher (McChesney *et al*., 1995); recharge of groundwater is altered

(Carbon *et al*., 1981) and less leaf litter (Ward and Koch, 1996) means less nutrient recycling and subsequently lower levels of soil microbial biomass. Data collected while studying the aetiology of the pathogen in a forest ecosystem can be expected to differ from that recorded in an open mine site (McChesney *et al*., 1995).

Detailed accounts of each stage of the clearing, mining and land rehabilitation process have been published (Ward *et al*., 1996; Colquhoun and Hardy, 2000) and an overview is given in Section 1.3 of this thesis. If the subsurface clay is compacted, ponding can result in the riplines after heavy rainfall (Fig. 5.2). Stems and lower branches can be submerged in these ponds which provide conditions conducive to the reproduction of *P. cinnamomi* (Zentmyer, 1980; Hardy *et al*., 1996) and transport of its infective propagules. *P. cinnamomi* is recognized as a soilborne pathogen, infecting roots of susceptible plants (Zentmyer, 1980) but when it is present in the ponded riplines of rehabilitated mine sites, pathogenesis can occur in the collars of plants (Hardy *et al*., 1996; O'Gara *et al*., 1997).

The inoculation technique chosen for the current study, a large field trial on a refilled bauxite mine site at Jarrahdale, Western Australia, was developed in the glasshouse (Chapter 4). It simulates infection of wet stems in ponded conditions and is an alternative to the non-wounding zoospore-inoculation method used by O'Gara *et al*., (1996). Previous forest-based research had used conventional wounding techniques to inoculate the plants (Tippett *et al*., 1985; Shearer *et al*., 1987; Davison *et al*., 1994; Bunny *et al*., 1995).

In *E. marginata*, disease development is rapid in plants with high bark moisture (Tippett and Hill, 1983). It is possible that, in the hot dry summers of the south-west of Western Australia, reduced plant moisture content may slow the infection process. This may allow enough time for the defence system of *E. marginata* to contain the pathogen with lignified tissue, particularly in *E. marginata* plants selected as genetically resistant to *P. cinnamomi* (Cahill *et al*., 1993). Increased plant moisture after abnormally high summer rainfall, as experienced in 1982, could facilitate both the infection process and the breakout of *P. cinnamomi* from contained lesions (Tippett and Hill, 1983) and the consequent death of the host plant.

Figure 5.1 Lack of canopy cover in a rehabilitated mine site adjacent to jarrah forest, prior to revegetation of the site.

Figure 5.2 Lower branches of *E. marginata* seedling in contact with ponding in a ripline in a rehabilitated mine site.

The aims of this field trial were to determine (1) if drought-stressed *E. marginata* plants are less susceptible to infection by *P. cinnamomi* than non-stressed plants in rehabilitated mine site conditions and (2) if the development of the disease caused by *P. cinnamomi* is more rapid in irrigated, non-stressed plants.

The null hypothesis was therefore,
H_o: There will be no difference between irrigated and droughted treatments in the development of the disease caused by *P. cinnamomi* in *E. marginata* plants in rehabilitated mine site conditions.

This experiment was set up in July, 1998, on an uninfested area of rehabilitated bauxite mine site. It will be seen that the impact of unseasonally high summer rainfall in January 2000 (Bureau of Meteorology, WA) rendered the original experimental design invalid but the field trial continued to be monitored for twelve months. This gave an insight into the potential for disease development after a summer rainfall event and its impact on *E. marginata* plants, even those resistant to *P. cinnamomi*. After the deluge, the irrigated and droughted plots were not considered separately but comparison was made between inoculated and non-inoculated plants. The rainfall caused local flooding and the movement of *P. cinnamomi* from an adjacent infested area of the rehabilitated mine site. This provided a rare opportunity for observations to be made of the natural spread of the pathogen.

5.2 Methods

5.2.1 *Experimental design*

Six hundred *E. marginata* plants, of a clonal line (77C40) resistant to *P. cinnamomi* (M. Stukely, *pers. comm*.), were planted in a used mine site prepared for rehabilitation and designated *P. cinnamomi*-free by the Environmental Department of Alcoa World Alumina (S. Jarvis, *pers. comm*.). There were 4 treatments with 50 replicates in 3 blocks. The blocks were split into 2 plots, one irrigated (non-stressed plants) and the other left to experience summer drought (drought-stressed plants). Half the plants in each plot were inoculated with *P. cinnamomi* and the others were sham-

inoculated as controls. Plants were inoculated in November 1999, after the winter and spring rains and prior to the anticipated summer dry period. Sixty plants were harvested each month for 10 months (Table 5.1).

Table 5.1 Protocol for the planting and harvest of drought-stressed (droughted) and non-stressed (irrigated) plants of a clonal line (77C40) of *Eucalyptus marginata* inoculated, when eighteen months old, with *Phytophthora cinnamomi*.

Block and plot number	Droughted or Irrigated	Inoculated or Control	Treatment code	Number of plants in treatment	Number of plants in each of 10 harvests
1 – plot 1	Droughted	Inoculated	D1	50	5
1 – plot 1	Droughted	Control	C1	50	5
1 – plot 2	Irrigated	Inoculated	B1	50	5
1 – plot 2	Irrigated	Control	A1	50	5
2 – plot 1	Droughted	Inoculated	D2	50	5
2 – plot 1	Droughted	Control	C2	50	5
2 – plot 2	Irrigated	Inoculated	B2	50	5
2 – plot 2	Irrigated	Control	A2	50	5
3 – plot 1	Droughted	Inoculated	D3	50	5
3 – plot 1	Droughted	Control	C3	50	5
3 – plot 2	Irrigated	Inoculated	B3	50	5
3 – plot 2	Irrigated	Control	A3	50	5

Close proximity of plants meant that droughted and irrigated treatments could not be allocated at random to plants within each plot. Plots 1 and 2 in each block were divided by a 4 metre buffer zone to separate the irrigated and the droughted plants and to ensure that the droughted plants, which were planted on the higher area of a very slight gradient, did not benefit from the watering system. Because of this limitation, the design was not a completely randomized block design, but within each plot of 100 plants (Fig. 5.3), 50 were inoculated at random with *P. cinnamomi*.

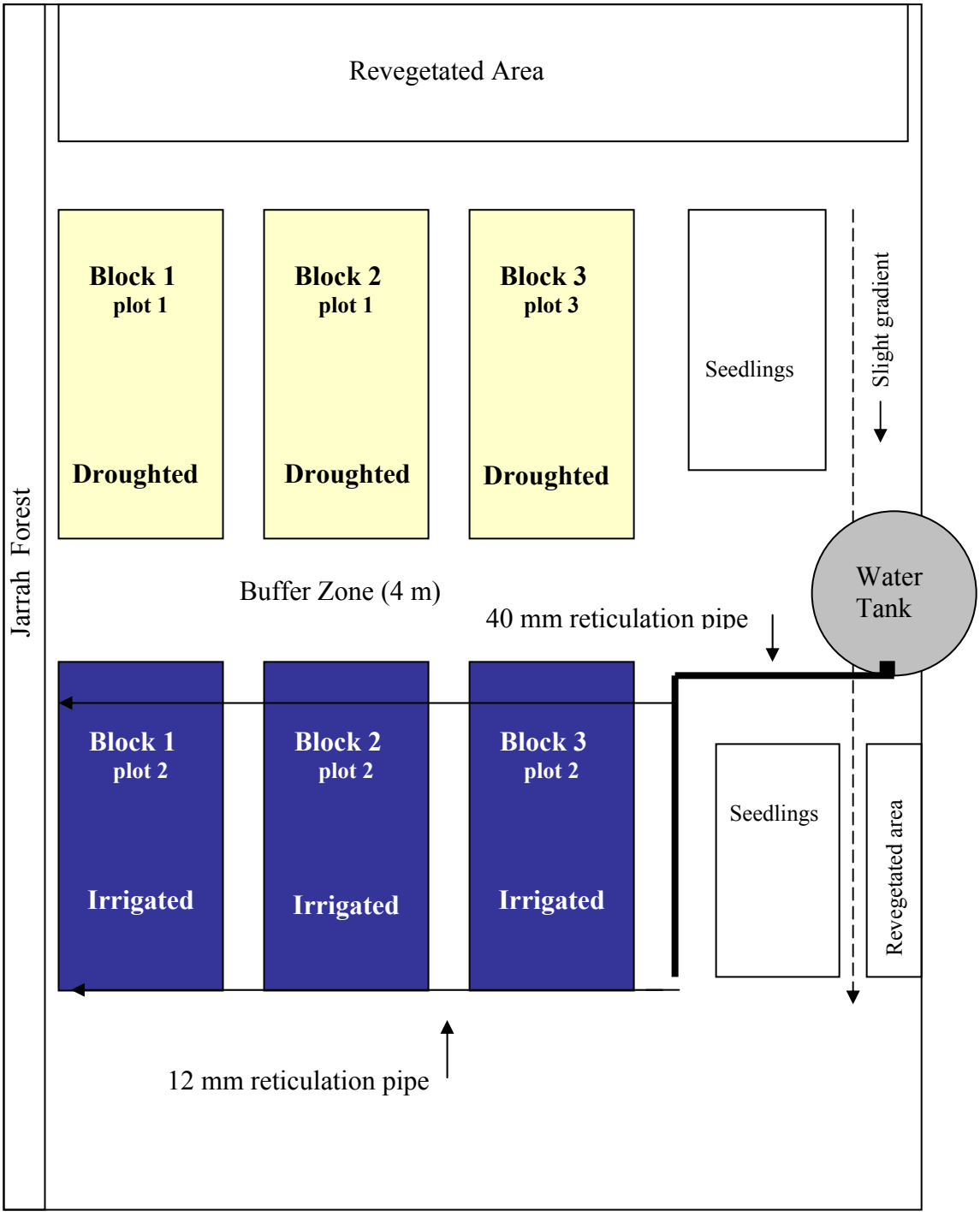


Figure 5.3 Diagram (not to scale) of the field trial. Irrigated plots were separated from droughted plots by a 4 metre buffer zone. An elevated tank, fitted with a timed water release program, supplied water via 40mm polyvinylchloride (PVC) pipeline to the irrigated plots. Fourteen lines of PVC 12mm reticulation pipe (2 of the 14 shown, arrowed) serviced the irrigated plots.

5.2.2 *Choice of site*

A refilled mine pit, Gull Pit, at the Jarrahdale bauxite mine of Alcoa World Alumina Australia, Alcoa map reference J38 10, where topsoil had been ripped with a deep and winged tyne in preparation for revegetation of the site, was chosen (Fig. 5.1). Parallel riplines, about 20-30 cms deep and 1 metre apart, extended for the length of the site. A stockpile of overburden soil nearby provided the >4 metres elevation needed for the installation of a water tank (Fig. 5.4) to provide adequate water pressure to the reticulation system. The site for this field trial was designated *P. cinnamomi*-free by Alcoa's environmental officers (S. Jarvis and Dr. I. Colquhoun, *pers. comm.)* Adjacent jarrah forest and revegetated areas (Fig. 5.3) were not. This site had a Havel classification of P/W (Dr. I. Colquhoun, *pers. comm*; Havel, 1975a, cited by Shearer and Tippett (1989) and soil was a coarse gravelly mix of clay and stones.

5.2.3 *Plant material*

Six hundred tube stock of six-month-old *E. marginata* (jarrah) plants of a clonal line (77C40), resistant to *P. cinnamomi* (M. Stukely, *pers. comm*.) were supplied by the Marrinup nursery of Alcoa World Alumina Australia. All were planted in July 1998, when ambient air temperatures were 4.9^{o}C to 14.5^{o}C. The monthly rainfall of 104.6 mm followed by 256 mm in August 1998, helped their establishment. These were planted on the ripline mounds with three 50g pellets of diammonium phosphate fertilizer positioned around each plant, a few centimetres from the root system, to a depth of 20 cm.

At the same time, 406 six-month-old tube stock *E. marginata* seedlings were planted in 2 adjacent plots (Fig. 5.3), in preparation for another field trial. These were later to be the first indicators of an outbreak of *P. cinnamomi* in the soil and its spread after summer rainfall.

5.2.4 *Water tank and reticulation system*

A 20,000 litre steel water tank (Jarrahdale Tanks, Jarrahdale, Western Australia 6203) was installed on the pile of overburden soil, 120 metres from the site, with >4 metres elevation to gravity feed the attached reticulation system (Fig. 5.4). Lines of 12

mm polyvinylchloride tubing were secured along the rows of *E. marginata* plants on the ripline mounds in the irrigated plots (Fig. 5.5) and drippers were inserted along the lines to water individual plants. An electronically controlled program using solenoid valves regulated the watering regime. Plants were irrigated for 2 hours per day, 3 days a week, with each dripper dispensing 2 litres per hour. This ensured that the irrigated plants were not water deficient. The tank was refilled fortnightly (Fig. 5.4).

5.2.5 *Inoculum preparation*

An axenic culture of *P. cinnamomi* isolate MU 94-48 was prepared after re-passaging through an *E. marginata* seedling (Appendix 9). Inoculum was prepared 7 days prior to the day of inoculation, in 90 mm Petri dishes, each containing 20 ml of sterile V8 nutrient agar (Appendix 2a). A plug of the axenic culture was placed in the centre of each plate, which was then sealed with Parafilm™ and incubated in the dark at 24±1^{o}C. After 7 days, mycelium had colonized the entire surface of the agar. A 1 cm x 1 cm grid placed below the plate ensured uniform agar plugs of inoculum were cut for application to each plant. Plates of sterile V8 agar were prepared as sham inoculum for control plants. The surfaces of all plates were covered with opaque tape to shield inoculum from direct sunlight in the open mine site. Polystyrene containers insulated the plates during transport to the mine site and while technicians moved between plants.

5.2.6 *Pre-treatment of stems*

The non-wounding technique required pre-treatment of stems three days prior to inoculation (Chapter 4). Cotton wool, soaked in distilled water, was autoclaved at 121^{o}C for 20 minutes for 3 consecutive days. All stems were pre-treated with sterile wet cotton wool, applied to the periderm area, about 8 cm above the soil line. The more sheltered southerly aspect was chosen as the application zone. The cotton wool was secured at the intended region of inoculation (ROI) with a protective wrap of Parafilm (Fig. 5.6). In the warm, open field conditions, extra flagging tape was wrapped around the stem and over the Parafilm to prevent the splitting of the Parafilm and subsequent drying out of the cotton wool (Fig. 5.7).

Figure 5.4 Elevated water tank, used for irrigation, being refilled at the mine site.

Figure 5.5 Lines of PVC tubing were used to irrigate some plots of *E. marginata* plants in the rehabilitated mine site.

Fig. 5.6

Fig. 5.7

Fig. 5.8

Fig. 5.9

Fig. 5.10

Fig. 5.11

Figure 5.6 Pretreatment of stems with wet cotton wool, held with Parafilm™ just before the protective flagging tape was wrapped around.

Figures 5.7 and 5.8 Flagging tape, Parafilm and cotton wool were removed immediately prior to inoculation.

Figure 5.9 Inoculum, agar colonized with *P. cinnamomi*, was placed on clean, wet cotton wool with a spatula, before application to the stem.

Figure 5.10 Parafilm was wrapped around the stem to secure the inoculum and the cotton wool.

Figure 5.11 A wrap of silver ducting tape gave additional protection against the weather.

5.2.7 *Inoculation*

Pre-treatment wraps were removed with a clean razor blade (Fig. 5.8). Using a spatula, a square of colonized agar was placed mycelial surface up on autoclaved wet cotton wool (Fig. 5.9). This was applied, mycelial surface to the periderm, on the moistened area of the stem, then secured with a wrap of Parafilm (Fig. 5.10). To eliminate strong sunlight from the ROI and to avoid desiccation of the inoculum and the cotton wool, a protective wrap of silver ducting tape was applied over the Parafilm (Fig. 5.11). Surplus inoculum from each plate was sub-cultured in the laboratory the following day, to confirm its viability after exposure to field conditions during the period of inoculation. All items were removed from the stem after 21 days and the ROI was marked with a permanent white marker pen (Mitsubishi Pencil Co. Ltd., Japan).

5.2.8 *Plant growth*

Heights of all plants, and stem widths at 10 cms above soil level, were recorded on the day of inoculation. Plant height was measured again at the December, January, February and April harvests and growth, relative to the height at inoculation, was calculated (Equation 5.1).

$$\text{Equation 5.1: Relative plant growth} = \frac{(\text{Height 2} - \text{Height 1})}{\text{Height 1}} / \text{days to death or harvest}$$

5.2.9 *Monitoring stress levels*

On the day prior to each monthly harvest, pre-dawn and mid-day readings of xylem pressure potential were taken, with a pressure chamber similar to that described by Scholander *et al*. (1965), of all plants (n = 60) to be harvested. Small branchlets of young leaves, taken from the same aspect of each plant, were insulated in polystyrene containers during transport and inserted into the pressure chamber within a few minutes of excision. On the same day, stomatal conductance was determined mid-morning and mid-afternoon using a steady state porometer (model LI-1600, LI-COR, Ltd. Nebraska, U.S.A.), Deaths prior to each harvest and general appearances of plants were noted.

5.2.10 *Stem moisture*

At each harvest, 3 x 1 cm segments were cut from each of 4 lateral branches from the 60 plants. Sets of 3 segments were put into 240 pre-weighed phials and fresh weight (FW) recorded. Phials were filled with distilled water and segments left immersed overnight. Excess water was removed with absorbent paper towels and turgid weight (TW) of the 3 segments was recorded. They were oven dried at 60^{o}C and after 3 days, or when no further moisture loss was noted, dry weight (DW) was recorded. Relative water content (RWC) was calculated (Equation 5.2, Duniway, 1976) and correlated with xylem pressure potential.

Equation 5.2:
$$RWC = \frac{(FW - DW)}{(TW - DW)} \times 100$$

5.2.11 *Soil moisture*

Soil samples were taken from each plot in October, the month prior to inoculation, and at each harvest. Five soil samples were taken from each of the 6 plots, close to where plants had been harvested. Gravel was removed from the soil, using a 3mm wire grid sieve. Fresh weight (FW) of 100g of soil from each sample, in pre-weighed paper bags, was recorded and the samples were dried in an oven at 60^{o}C for 7 days. Dry weight (DW) was recorded and the soil moisture, expressed as a percentage of FW was calculated (Equation 5.3).

Equation 5.3:
$$\text{Soil moisture}\,(\%) = \frac{(FW - DW)}{} \times 100\%$$

5.2.12 *Rainfall and temperature*

Data were provided by the Bureau of Meteorology from Station 9111 at Karnet (latitude 32°26'24"S; longitude 116°04'28"E) the closest weather station, approx. 15 km from the field site, that records rainfall and temperatures. Rainfall data for the previous 35 years were provided in calendar month totals. Comparison has been made of the historic median monthly rainfall with the actual monthly rainfall during this trial (Fig. 5.12a). Ambient temperatures recorded during the trial are presented as the mean for each calendar month (Fig. 5.12b).

A separate calculation was made for monthly rainfall prior to each harvest, because rainfall after each monthly harvest affected the data for the following harvest. Daily rainfall data were provided for the twelve months that the field trial was monitored (October to September). For comparisons between harvests, and for correlation of rainfall to other observations, the monthly rainfall (mm) data are presented as rainfall after the previous harvest date. i.e. prior to and including the current harvest date or for the 30 days preceding the harvest. For example, prior to the first harvest on November 25, total rainfall (1.0 mm) is the sum of all rainfall in the 30 days prior to the harvest date (Fig. 5.13). In addition, data loggers (Starlog, Unidata Australia, O'Connor, Western Australia 6163) housed in waterproof boxes and with probes to measure soil temperature and ambient temperature at the site, were installed. A rain gauge was installed on site to provide confirmation of rainfall.

5.2.13 *Summer rainfall event*

The northern jarrah forest normally experiences summer drought for approximately 6 months from October to April/May, with high winter rainfall from May to September (Fig. 5.12a). In January 2000, prior to the January harvest, three major unseasonal summer rainfall events occurred with attendant thunderstorm activity. Figures provided by the Bureau of Meteorology document a monthly rainfall twenty times the January median of 5.6 mm (Fig. 5.12a). This unseasonal event rendered the hypothesis for the

field trial invalid. The treatments (watering regimes) were negated by the sudden deluge. Ponding was observed in the flooded riplines, and persisted for several weeks, especially in the lower lying area of the seedling plot (Fig. 5.14). The ponding and the warm weather provided favourable conditions for the outbreak and spread of *P. cinnamomi* already present in a neighbouring rehabilitation area (Fig. 5.3). The seedlings were first affected and the advance of the pathogen through the seedling plot and the trial area was mapped from January to August, 2000 (Figs 5.22a and 5.22b).

Samples of diseased plants from the three separate areas of the mine site (1) the field trial with clonal plants, (2) the seedling plot and (3) the two-year-old rehabilitation area were collected for plating onto NARPH selective agar and for molecular analysis.

5.2.14 *Harvests and continued monitoring of the site*

Two harvests, November and December 1999, were made 21 and 48 days after inoculation, respectively, and before the summer rainfall event (5.2.13). Stems were cut as close to the soil surface as possible. Surface lesions were measured and, after samples had been taken for stem moisture (5.2.10), side branches were removed and the stems cut into 0.5cm segments with a bandsaw from 20 cms above the lesion front and down to it. If no lesion was visible, segments were cut from 20 cms above the ROI and down to it. Segments were cut into smaller pieces with secateurs, which were flamed, after immersion in 70% ethanol, to avoid cross-infection. These pieces were plated onto NARPH agar, selective for *Phytophthora* (Hüberli *et al*., 2000). Plates were sealed with Parafilm and incubated in the dark at 24±1^{o}C. Daily monitoring with a dissecting microscope (magnification x 70) confirmed the presence or absence of *P. cinnamomi*. Infected segments and surrounding colonized agar were removed with a sterile scalpel before the plate was returned to the incubator. This prevented overgrowth and the masking of emergent hyphae from other segments. Monitoring continued for 14 days. Lesion lengths and colonization beyond the lesion or beyond the ROI were recorded.

Despite the unexpected summer rainfall event, and the subsequent negation of treatments, harvests and other observations were continued. Full harvests of 60 plants were made after this in January, February and April, with additional monitoring in

March, June and August, 2000. In June and August, stem sections – cutting through the periderm and phloem to the cambial zone - were taken *in situ* from one side of the stem at the ROI and above for 4 - 6 cms, then plated onto NARPH, to monitor the number of plants infected and proportion of plants from which recoveries of *P. cinnamomi* were made over time. Inoculated stems from which no *P. cinnamomi* was recovered were subjected to further treatments. Segments were immersed in sterile distilled water or soil filtrate to leach out inhibiting phenolic compounds (Chapter 9) and replated onto NARPH agar.

5.2.15 *Statistical analysis and data presentation*

Following the original experimental design (5.2.1), data were initially analysed to determine the effect of drought on disease development in the plants by comparing the results of observations of plants in droughted plots to those of irrigated plots. The unseasonal summer rainfall (Fig.5.12a), which occurred soon after inoculation, negated the planned effect before enough time had elapsed after spring rains to establish droughted conditions. When, for most observations, no significant ($P > 0.05$) difference was detected between droughted and irrigated plots, these data were pooled and ANOVAs calculated of inoculated plants and non-inoculated plants.

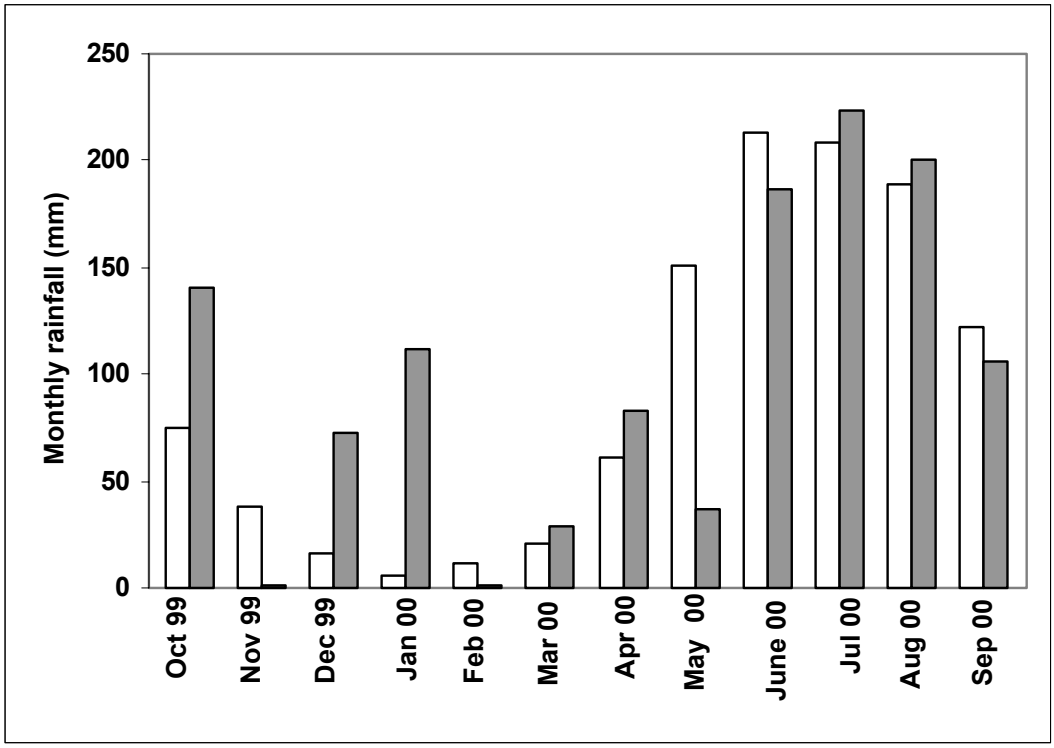
Data were analysed using Statistica® for Windows Version 5 (StatSoft Inc., OK, USA) and Excel for Windows (Microsoft Corporation, USA), Microsoft Excel Analysis ToolPak (GreyMatter International, Inc., Cambridge, MA 02141). Prior to parametric analyses, data were screened for basic assumptions of parametric tests including normal distribution, equality of variances and non-correlation of means and variances. In cases where data did not conform to assumptions, log transformations, as noted, were used to correct the deviations. Where data were log-transformed, means are presented graphically for non-transformed data. After comparison of data between or within treatments, where an ANOVA resulted in a P-value of < 0.05, data were considered significantly different.

5.3 Results

5.3.1 *Rainfall and temperatures*

The historic medians for each month for rainfall recorded over 35 years (Bureau of Meteorology, Karnet) are typical of a mediterranean climate with cool wet winters

and hot dry summers. Comparison has been made of the historic median monthly rainfall with the rainfall for each calendar month during this trial (Fig. 5.12a). This shows the huge increase in summer rainfall in December, 1999 and January, 2000. Ambient temperatures recorded during the trial are presented as the mean for each calendar month (Fig. 5.12b). Rainfall in the month prior to each harvest, i.e. from the previous harvest date up to and including the monthly harvest, or for 30 days prior to the harvest date or date of monitoring (as in June and August) shows that this mostly affected the January and February harvests (Fig. 5.13).



with

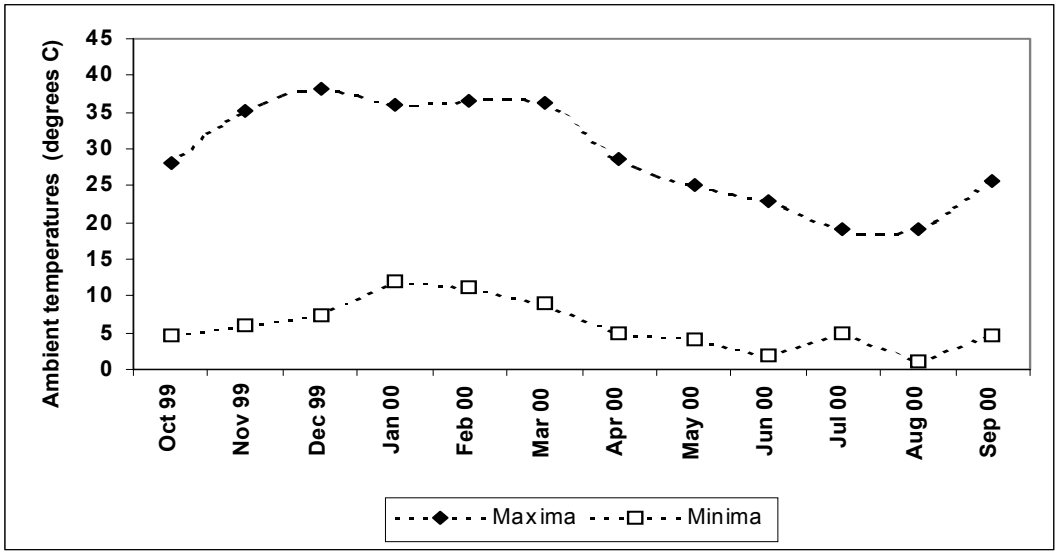


Figure 5.12b Ambient air temperatures during the field trial.

a

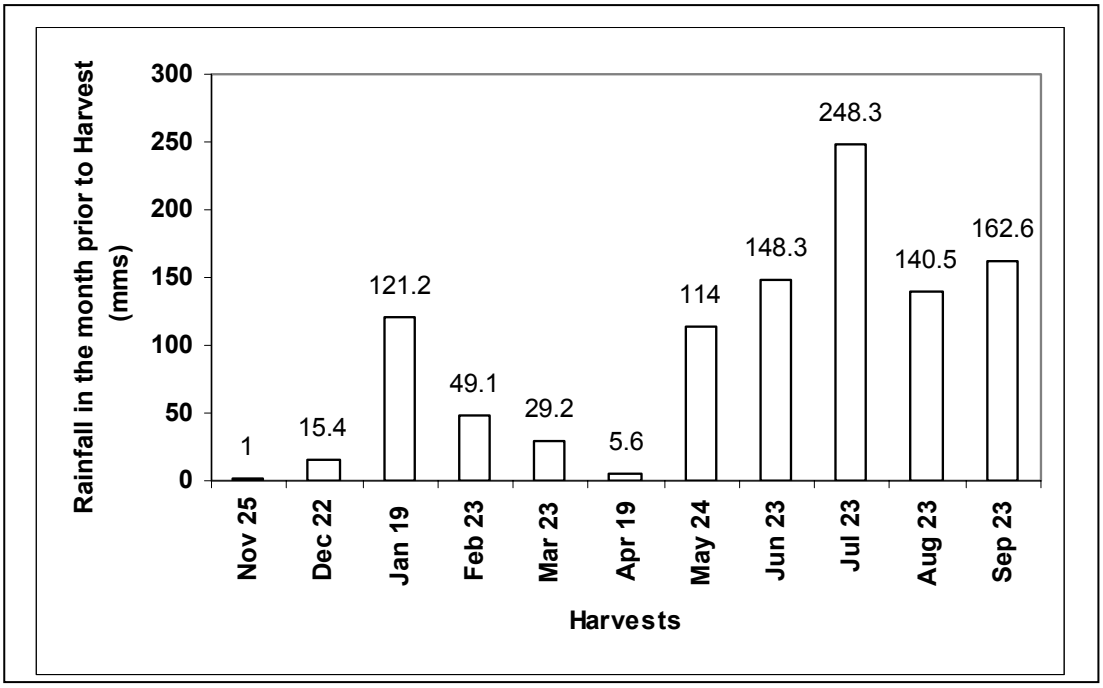
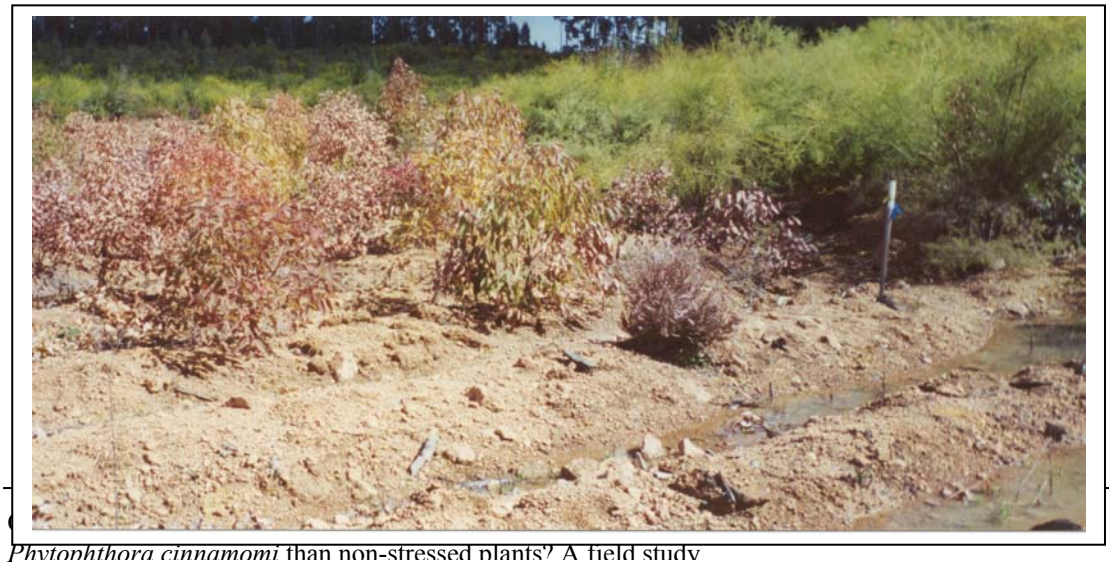


Figure 5.13 Total rainfall (mm) in the month prior to each harvest or time of monitoring of site, i.e. after the previous harvest and up to and including the current harvest date. Rainfall after each harvest affects the next monthly harvest.



Phytophthora cinnamomi than non-stressed plants? A field study

Figure 5.14 Ponded riplines and dying *E. marginata* seedlings adjacent to a revegetated area of the mine site.

Data could not be retrieved from the data loggers, possible due to a lightning strike, and the capacity of the rain gauge was inadequate for the inundation.

5.3.2 *Summer rainfall event*

The South West Land Division of Western Australia, which includes the Jarrahdale/Karnet area, experienced 3 heavy rainfall events in January 2000, all of which were associated with thunderstorms produced by the approach of an upper level trough and low-level convergence in the easterly flow (Bureau of Meteorology). Unseasonally high summer rainfall in January (112 mm) (Fig. 5.12a) had a major impact on the field trial, negating the comparison of the droughted and irrigated treatment. Subsequent ponding in the riplines (Fig. 5.14), high soil moisture content (Fig 5.19) and the warm temperatures provided conditions conducive to the reproduction of *P. cinnamomi* and an outbreak of the pathogen in the soil, unrelated to the inoculation process, was observed (Figs. 5.22a and 5.22b).

5.3.3 *Plant growth*

Six of the 600 clonal plants died prior to inoculation. These were from Treatments A (irrigated controls, 1 plant), B (irrigated and to be inoculated, 2 plants), C (droughted controls, 1 plant) and D (droughted and to be inoculated, 2 plants). Replacements were planted in the same situations during early monitoring, but these also did not survive. Large rocks underlay the topsoil in places and it is likely that these were too great an impediment to root systems foraging for nutrients and moisture. No *P. cinnamomi* was recovered from these six plants.

At inoculation, plants were two years old. Mean height of all clonal plants (n = 594) on the day of inoculation was 99.51±SE 0.92 cm. There was no significant (*df* 3, 590; P >0.05) difference in heights between treatments. Mean stem width of all plants (n = 594) on the day of inoculation was 30.35±SE 0.38 mm. There was no significant (*df* 3, 590; P >0.05) difference in stem widths between treatments.

A 2-way ANOVA with inoculation status (inoculated or non-inoculated) and month of harvest (December, January, February and April) showed that only the inoculation status had a significant (P < 0.001) effect on relative plant growth. Inoculated plants grew less than control plants each month and there was a significant (*df* 1,58; P < 0.05) difference at the December and February harvests with a result close to significant (*df* 1,58; P = 0.07) at the January harvest (Fig. 5.15).

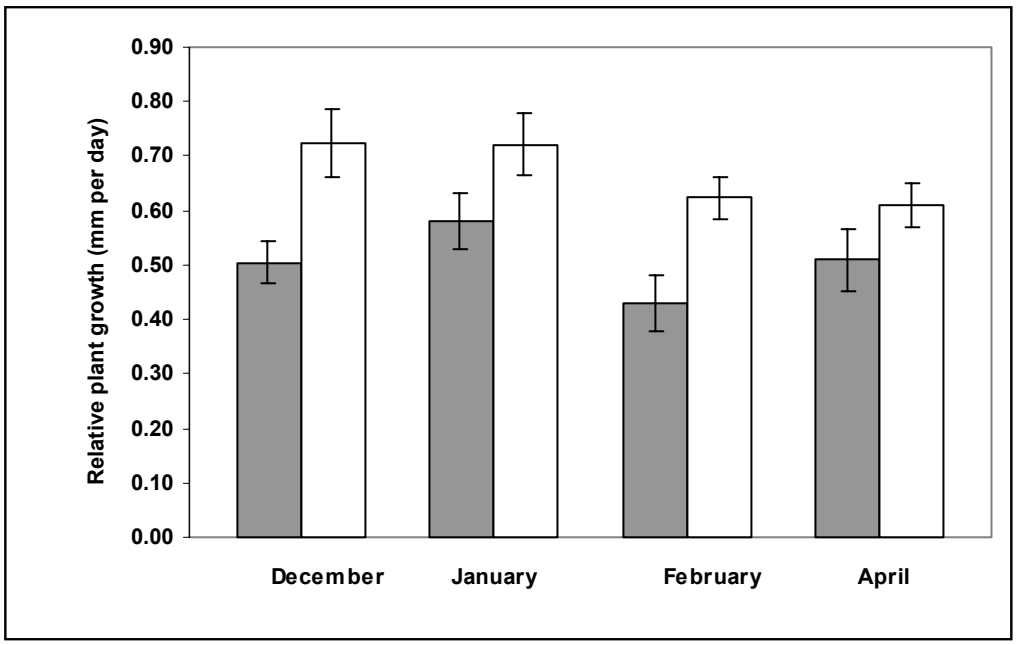


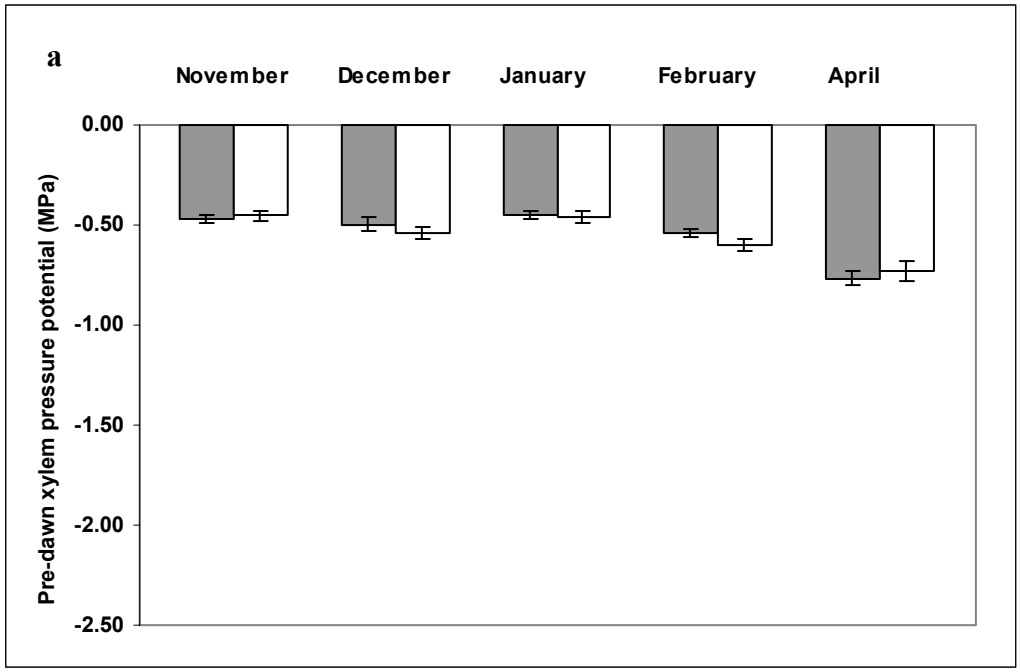
Figure 5.15 Growth of inoculated and control plants at 4 harvests, relative to the height at the time of inoculation in November. Bars represent the standard error of the mean.

Inoculated plants

Control (non-inoculated) plants

5.3.4 *Xylem pressure potential*

Mean pre-dawn xylem pressure potential (XPP) was always less negative than the mean midday XPP for both inoculated and control plants (Fig.5.16).



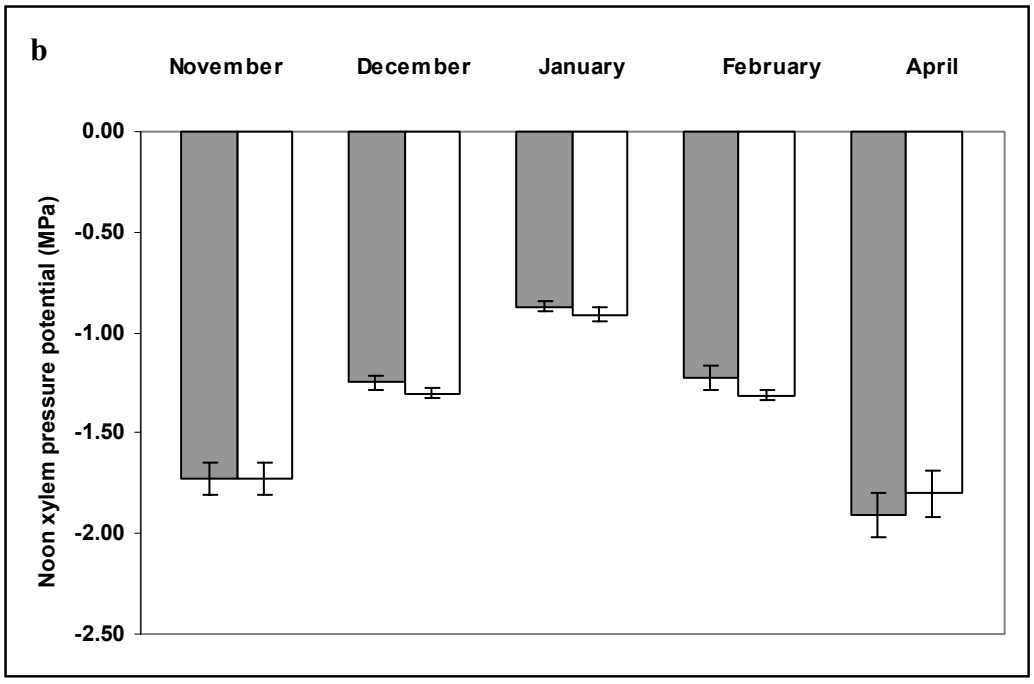


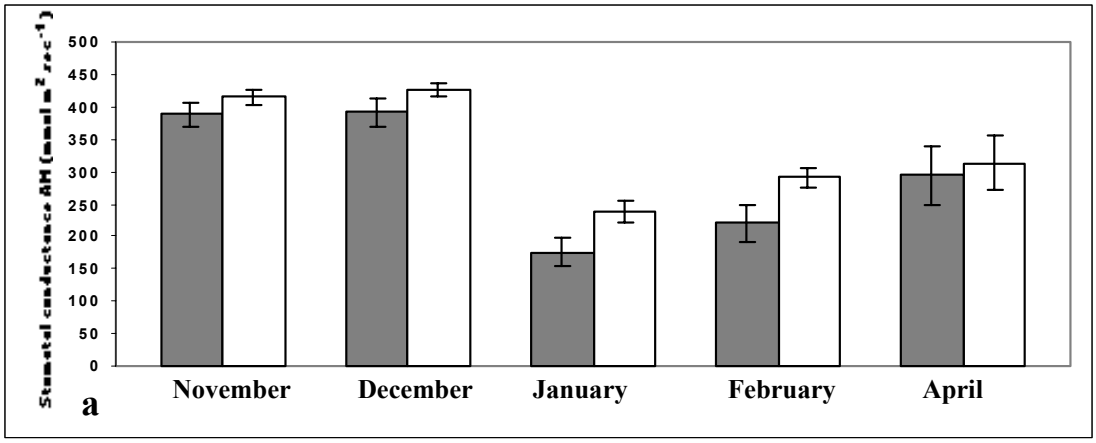
Figure 5.16 Mean xylem pressure potential of *E. marginata* (77C40) plants, (a) pre-dawn and (b) at noon, at each harvest. Bars represent the standard error of the mean.

Inoculated plants

Control Plants

5.3.5 *Stomatal conductance*

Stomatal conductance was always lower in inoculated plants than in non-inoculated plants. Two-way ANOVAs of inoculation status and month of harvest showed that readings for both morning (am) and afternoon (pm) stomatal conductance were significantly ($P < 0.001$) different between months, but within months, there was a significant difference only in am readings for January (*df* 1,55; P 0.02) and February (*df* 1, 52; P = 0.03) (Fig. 5.17).



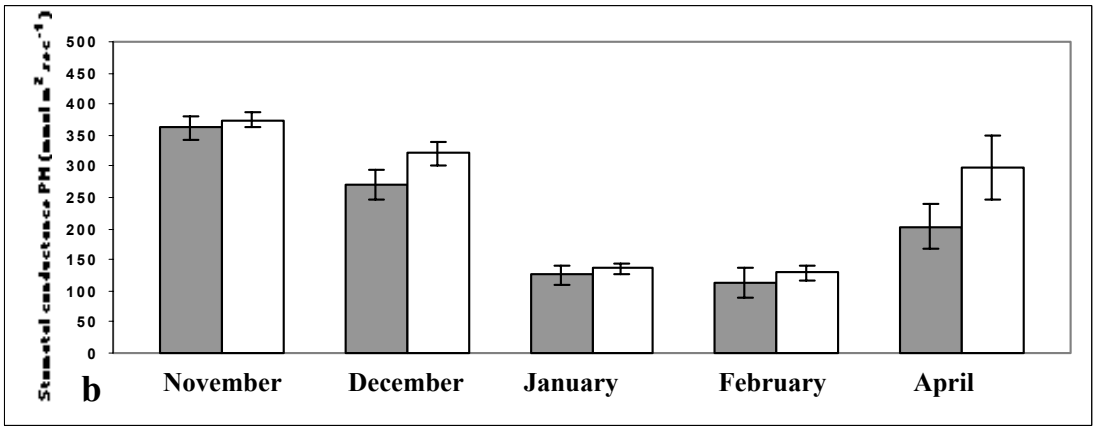


Figure 5.17 Stomatal conductance of *E. marginata* plants (a) am and (b) pm. Bars represent the standard error of the mean.

Inoculated plants

Control (non-inoculated) plants

5.3.6 *Stem moisture*

A 2-way ANOVA of log-transformed data showed that inoculation status had no significant ($P > 0.05$) effect on relative water content (RWC) but that there was a significant ($P < 0.05$) difference in results between harvest months. There was no significant ($P > 0.05$) difference in inoculated plants between months but a significant (*df* 4, 123; $P < 0.001$) difference in RWC in control plants between months (Fig 5.18). Data for dead plants were omitted but outliers for disease-affected plants were included.

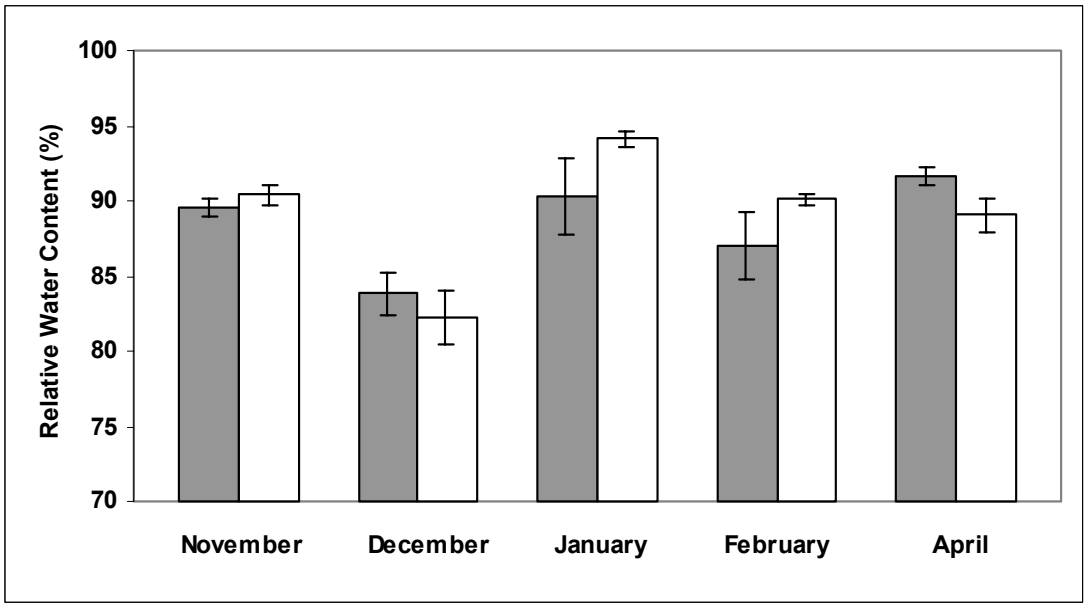


Figure 5.18 Stem moisture expressed as relative water content in harvested stems of *E. marginata* (77C40), resistant to *P. cinnamomi*. Bars represent the standard error of the mean.

Inoculated plants

Control (non-inoculated) plants

5.3.7 *Soil moisture*

After winter and spring rains, the mean percentage of moisture in soils sampled in October, prior to inoculation of plants and prior to installation of the watering system, was 10.83±0.40. In the following months at harvest, mean percentages were lower than this in all plots, but almost the same in January after the summer rainfall. There was a significant (*df* 4, 139; $P < 0.001$) difference in soil moisture between the harvest months (Fig. 5.19).

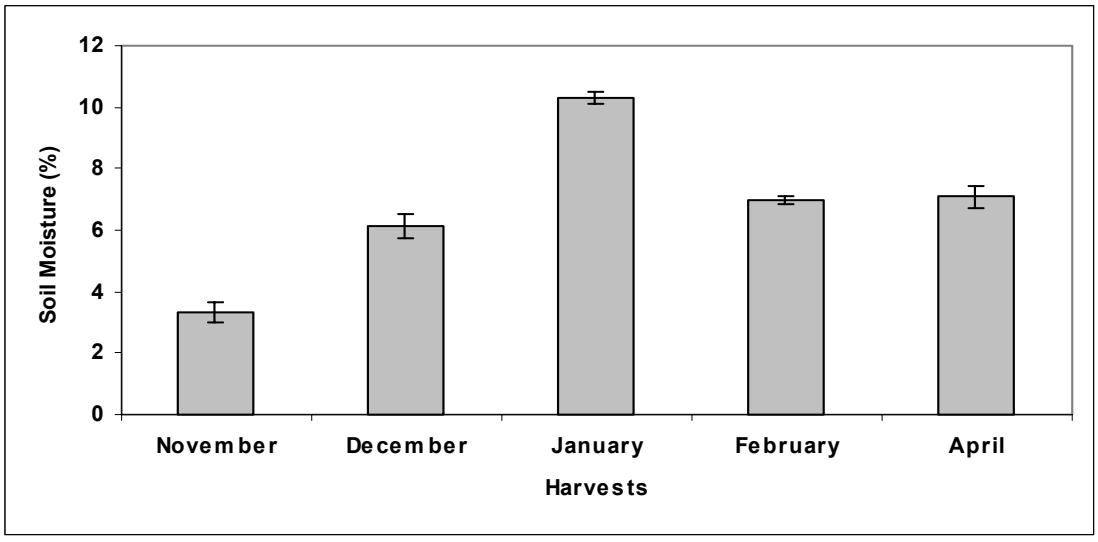


Figure 5.19 Soil moisture at time of each monthly harvest.
Bars represent the standard error of the mean.

5.3.8 *Recovery of* P. cinnamomi

In the first harvest, November 1999, most recoveries of *P. cinnamomi* were from the irrigated plants. Summer rainfall negated this treatment and recoveries from irrigated and droughted blocks have been pooled (Table 5.2). Re-plating, after immersion of stem segments in water to leach phenolic compounds from the tissue of infected stems, resulted in additional recoveries (Fig. 5.20). Over time, there was a decrease in recoveries from direct plating and from re-plating after leaching (Fig. 5.20).

Table 5.2 Recovery of *P. cinnamomi* from inoculated *E. marginata* plants of a resistant clonal line 77C40.

Harvest	Rainfall in month prior to harvest (mm)	Plants harvested or sampled *n* =	Recovery from direct plating *n* =	Total recovery after leaching *n* =	Recovery from direct plating %	Total recovery after leaching %
November	1.0	30	21	22	70.00	73.33
December	15.4	30	9	9	30.00	30.00
January	121.2	30	12	12	40.00	40.00
February	49.1	30	13	16	43.33	53.33
April	5.6	30	10	13	33.33	43.33
June	148.3	41	16	19	39.02	46.34
August	140.5	31	0	3	0.00	9.68
Totals		195	84	97	43.08	49.74

Rainfall is not for the calendar month, but is the total (mm) recorded from the harvest date in the previous month up to and including the current harvest date (Fig. 5.13). If there is no previous monthly harvest, it is for the 30 days prior to harvest (November, June and August).

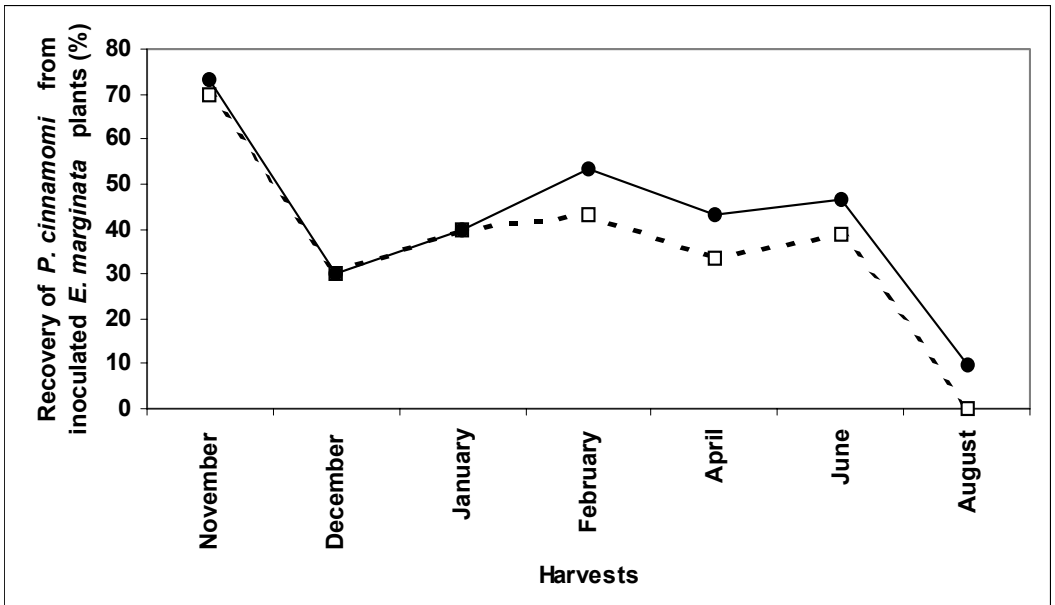


Figure 5.20 Decrease in recovery of *P. cinnamomi* from *E. marginata* (77C40) resistant plants over time, from November 1999 to August 2000, both with the direct plating (- - - - - -) and after leaching ( ).

5.3.9 *Lesion lengths and colonization*

The greatest extent of colonization was in the irrigated plants harvested in February after the summer rainfall event, which had lesions up to 60 cm. In all months but December, colonization was greatest in irrigated plants. No surface lesions were observed in the November, December or January harvests but recoveries were made from the non-lesioned stems. In contrast, from the 20 stems with surface lesions harvested in February and April, *P. cinnamomi* was recovered from only 14. Other recoveries were made from non-lesioned stems and additional recoveries were made after leaching and re-plating (Table 5.2).

A two-way ANOVA of log-transformed data showed that both treatment (irrigated or droughted) and month of sampling (November to April) had no significant ($P > 0.05$) effect on total colonization of harvested plants. There was a considerable increase in disease development in February (Fig. 5.21), but after data for irrigated and droughted plants were pooled, a one-way ANOVA of log-transformed data showed no significant (*df* 4, 145; $P > 0.05$) difference between months.

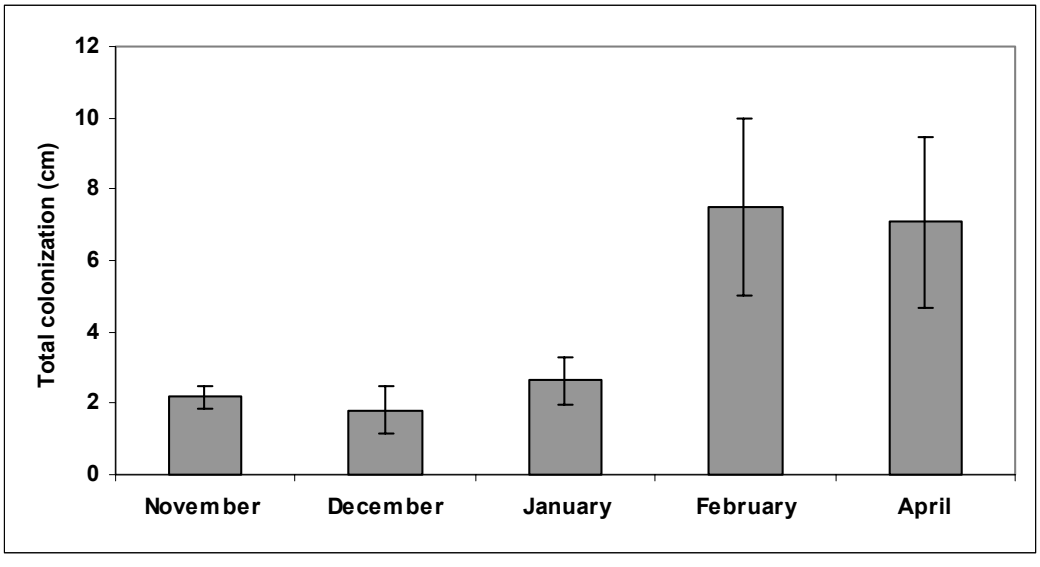


Figure 5.21 Mean total colonization of harvested *E. marginata* 77C40 plants, resistant to and inoculated with *P. cinnamomi*. Data from irrigated and droughted plants were pooled. Bars represent the standard error of the mean.

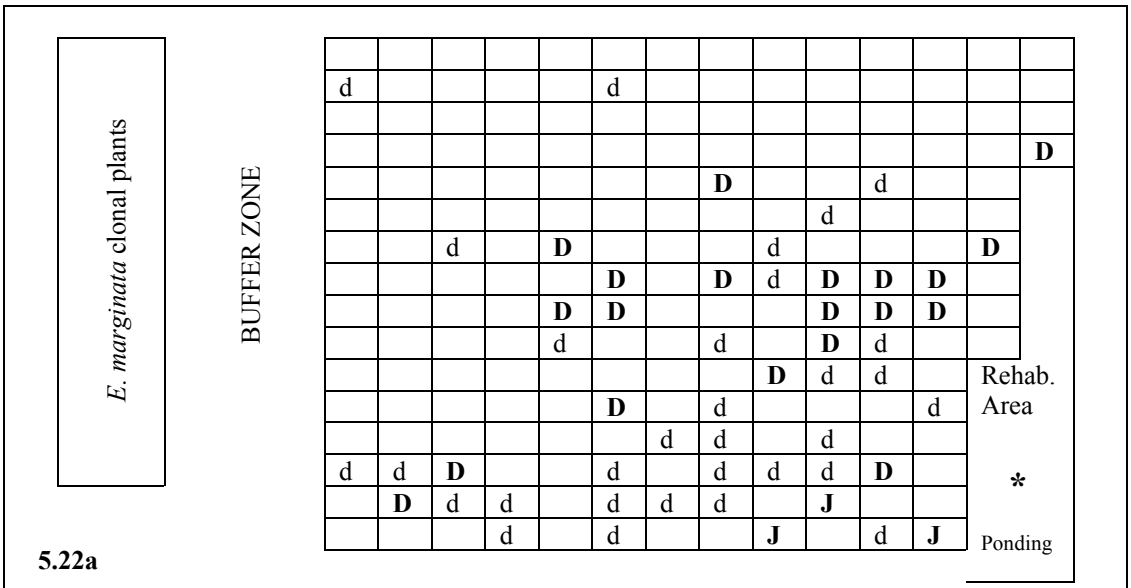
5.3.10 *Continued monitoring of the site*

After the summer rainfall event in January 2000, the monitoring schedule was altered and full harvests ceased after April. The site was monitored every two months. Two deaths of non-inoculated, clonal control plants were noted, one in February and one in April. Recovery of *P. cinnamomi* declined as indicated by the results of stem samples taken in June and August (Table 5.2). Equipment was removed and a final inspection of the site made in October, 2000.

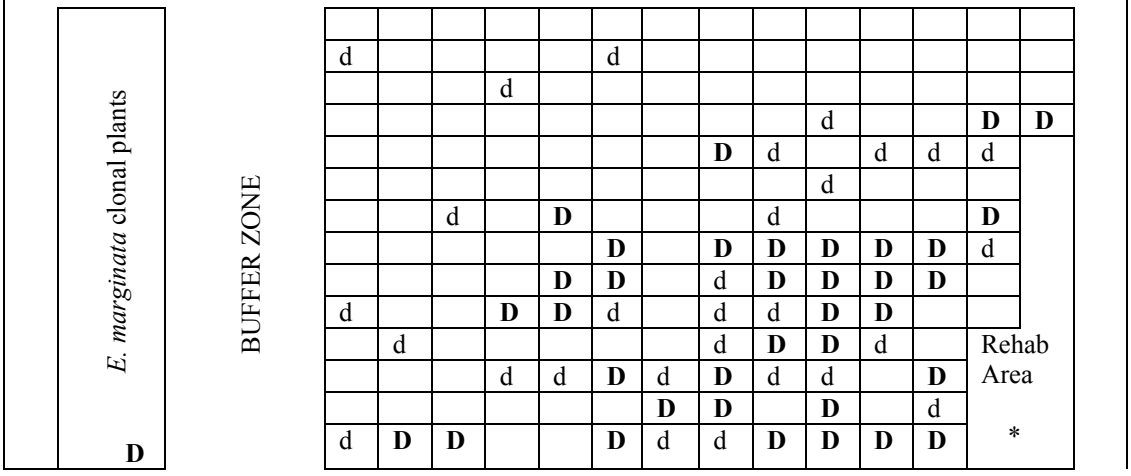
5.3.11 *Autonomous spread of* P. cinnamomi

After the heavy rainfall in January 2000, three plant deaths were noted in the seedling plot, which was located between a revegetated, rehabilitated area and the irrigated clonal plants (Fig. 5.3). Local flooding had resulted in intermittent ponding in the riplines throughout the trial area and ponding was observed near the dead seedlings. In February, more seedling deaths were noted and stem lesions, characteristic of *P. cinnamomi* were visible. A lesioned branch was taken from a dying *E. marginata* seedling in the nearby rehabilitated area (Fig. 5.22a) where other plant deaths suggested the presence of a plant pathogen. After plating segments of infected lignotubers, stems and branches onto selective agar, *P. cinnamomi* was recovered from *E. marginata* in the seedling plot and in the rehabilitated area. The death of an irrigated, non-inoculated clonal *E. marginata* control plant in February, was investigated and the plant was found to be also infected with *P. cinnamomi*. Molecular analysis indicated that the *P. cinnamomi* recovered from the clonal control plant in the field trial, the seedlings and the *E. marginata* in the rehabilitation area, was different from the isolate used to

inoculate the clonal plants in the field trial (Dr. M. Dobrowolski, *pers. comm*.). The seedling plot was monitored from January to August, 2000 and the advance of the pathogen mapped. By August, there were 44 dead and 35 dying seedlings, i.e. 38% of seedlings in the plot were affected (Fig. 5.22a and 5.22b). The lesioned stems of seedlings were harvested and used in later experiments to determine the effect of exudates of infected stems on the mycelial growth of *P. cinnamomi* (Chapter 9). There were, however, healthy seedlings, apparently more resistant to the pathogen, surrounded by dead and diseased plants (Fig. 5.22b) which could be the source of resistant clonal lines in future research.



5.22a



5.22b

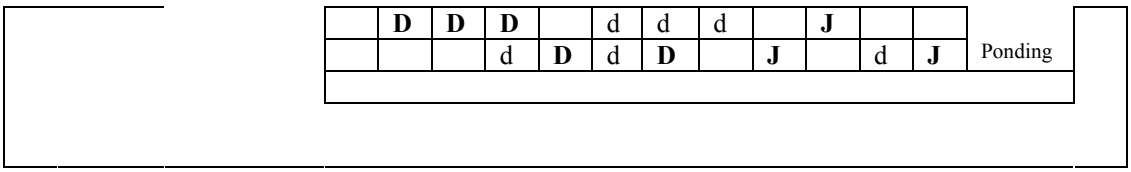
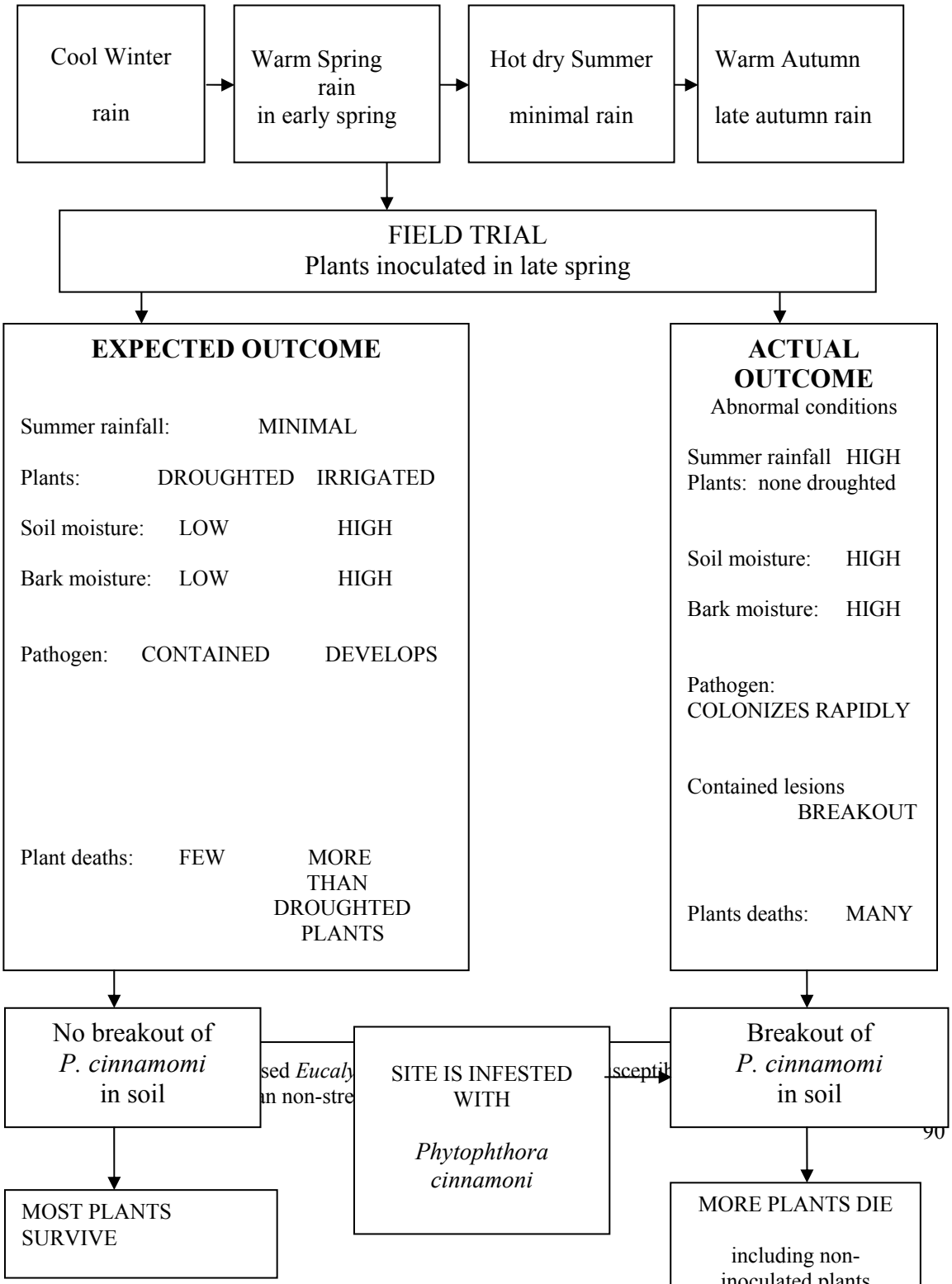


Figure 5.22a and b Diagram (not to scale) of the advance of *P. cinnamomi* in the soil as indicated by the increasing deaths of *E. marginata* seedlings from 3 deaths in January 2000, to 23 deaths in February 2000 (Fig. 5.22a) and 44 deaths by August 2000 (Fig. 5.22b). Each square represents a planted seedling (n = 206).
J = Dead seedlings in January (3 only).
D = Dead seedlings (and one clonal plant).
d = dying seedlings;
Rehab area = rehabilitation area. * = infected *E. marginata* in rehabilitated area.



5.4 Discussion

This field trial demonstrated the effects of abnormally high summer rainfall, in a region which normally experiences summer drought, on the development of disease caused by *P. cinnamomi* in *E. marginata* plants. This unseasonal event also highlighted the potential for inoculum, in soil or in already infected hosts, to spread rapidly in these conditions in a rehabilitated mine site.

5.4.1 *The summer rainfall events*

The original experimental design became invalid when the summer rainfall events negated the comparison of watering regimes. When no significant ($P > 0.05$) differences were observed between irrigated and droughted treatments, data were pooled. Another proposed field trial was abandoned when a high proportion of seedlings, planted in preparation (Fig. 5.3), became infected. However, continued recording of observations and site monitoring produced data which would not be available in a normal summer. Previous forest studies of *E. marginata* and *P. cinnamomi* have noted the effect of high rainfall on disease (Tippett *et al*., 1985; Bunny *et al*., 1995). To my knowledge, this is the first study to monitor the effect of high summer rainfall on both the spread of *P. cinnamomi* and the development of disease in plants in a rehabilitated mine site.

In January 2000, there were several rain-bearing systems which contributed to above average monthly rainfall in the Southwest Land Division of Western Australia. A middle level trough to the west of the state interacted with a surface trough to produce widespread thunderstorms, heavy rainfall and localised flooding, prior to the January harvest. The weather pattern was repeated and there was significant heavy rainfall in

the south-west with a number of localities reporting the highest falls on record or more than 100 mm of rain (Bureau of Meteorology, W.A.). The rainfall events were before the January and February harvests and would have contibuted to the outbreak of *P. cinnamomi* in the soil.

5.4.2 *Lesions and recovery of* P. cinnamomi

The effect of the summer rainfall events was reflected in the extent of colonization by *P. cinnamomi* in the harvested plants. After the normal winter and spring rains, there was insufficient time for droughted conditions, normally experienced in the south-west of Western Australia, to develop prior to the higher than normal rainfall in December and the extreme rainfall in January. The percentage of soil moisture increased in December and was highest in January. Coupled with the warm temperatures, this provided conditions which favoured the development of disease (Zentmyer, 1980; Shearer and Tippett, 1989).

There was a substantial increase in the total colonization of stems in the February harvest and a corresponding increase in February of the relative water content of the plants. Fewer recoveries of *P. cinnamomi* and less colonization of stems in December, suggest containment of the pathogen by the resistant host. However, after the extreme rainfall events, there was an increase in pathogen activity as indicated by the increase in both recovery of and colonization by the pathogen in February. Previous studies have reported the breakout of contained *P. cinnamomi* lesions in *E. marginata* after heavy summer rains (Tippett and Hill, 1983) and more growth of *P. cinnamomi* lesions in plants with higher bark moisture (Tippett and Hill, 1983) or higher water status (Bunny *et al*., 1995). Similarly, studies with *P. cinnamomi* in *Quercus* spp. found that root infections were less severe when plants were droughted than when intermittently flooded (Robin *et al*., 2001).

The percentage of inoculated stems from which *P. cinnamomi* was recovered was greatest (73%) in the first harvest 21 days after inoculation in November, declining to 33% in December, with increases in the subsequent harvests following the heavy rainfall. Recovery was not made from all lesioned stems, prompting the investigations to improve recovery methods (Chapter 9). Crombie and Tippett (1990) also reported few recoveries of *P. cinnamomi*, i.e. 10 from 23 lesioned stems of *E. marginata* in the

field. Recovery in the current field trial was not as successful as in the glasshouse pilot trial (Chapter 4). The same isolate of *P. cinnamomi* was used in both trials, but factors contributing to fewer recoveries would be that the plants in the field were clonal plants, resistant to *P. cinnamomi* (M. Stukely, *pers. comm.)* and as older, field-grown plants, they had a more developed periderm than the glasshouse-grown seedlings. Several researchers have noted a decline in recovery and colonization over time (Weste and Ashton, 1994; Bunny *et al*., 1995; Duncan and Keane, 1996; O'Gara 1998; McDougall *et al.,* 2002). Immersion of stem segments in water to leach out phenolic compounds, which inhibit the growth of *P. cinnamomi* (Chapter 9), improved overall recovery by 7%, supporting the findings of previous studies of *P. cinnamomi* recovery from *E. marginata* (O'Gara, 1998; Hüberli *et al*., 2000), which also found that leaching improved total recovery.

5.4.3 *Plant stress and responses to disease*

More negative xylem pressure potential or lower stomatal conductance indicate greater plant stress (Turner, 1981; Crombie and Tippett, 1990). Xylem pressure potential for both pre-dawn and noon readings was less negative in January, becoming more negative in February with less rainfall but increased colonization by the pathogen. The water potential of inoculated plants was not consistently more negative than the control plants, which does not agree with previous findings (Crombie and Tippett, 1990; Robin *et al*., 2001) but the plants in the current study were clonal plants, resistant to *P. cinnamomi* and the pathogen was not recovered from all inoculated plants, i.e. not all inoculated plants were necessarily infected, though all readings were included in data analyses. Most readings were less negative than – 1.5 MPa, at which *P. cinnamomi* growth in *E. marginata* can be inhibited (Tippett *et al*., 1987).

Stomatal conductance (both am and pm) was less in inoculated plants than in control plants at all harvests. This agrees with readings taken over two years of *E. marginata* infected with *P. cinnamomi* (Crombie and Tippett, 1990). Lower readings in both treatments in January and February do not appear to support the xylem pressure potential readings for January and February. Plants were not water stressed at this time and the lower readings for the inoculated plants may be attributed to stomatal closure because of the cytokinins produced by *E. marginata* in response to pathogenic invasion

(Cahill *et al*., 1986b). These phytohormones affect the stomatal conductance but not the xylem pressure potential. However, it would be expected that the readings for the control plants during January and February would be higher than the values recorded.

Plant stress was also indicated by a decrease in relative growth of the inoculated resistant clonal plants in February, suggesting an allocation of resources to plant defence rather than to growth in the inoculated plants. Reduction in growth was also noted in the control plants which, in conjunction with the lower stomatal conductance, suggests an additional, unobserved stress. This may have been root infection as the pathogen was spreading through the soil at this time. Defence responses may have activated phytohormonal activity and subsequent stomatal closure, without there being an obvious expression of the disease in the harvested stems of the control plants.

5.4.4 *The seedling plot*

Extensive lesions were also observed on the stems and branches of the seedlings in the field. These had been naturally infected after the summer rainfall events in January when riplines in the mine site were flooded. There was a slight gradient in the topography of the rehabilitated mine site and when three non-inoculated dead seedlings were first observed in low-lying areas after the heavy rainfall, waterlogging was the suspected cause of seedling death. The subsequent observation of stem lesions and the recovery of *P. cinnamomi* from the lignotubers and non-inoculated stems suggested root or collar infection An increase in rates of infection in low-lying sites has been noted (Shearer and Shea, 1987; Dell and Malajczuk, 1989) and waterlogging may be a contributing factor in disease development and death of *E. marginata* infected with *P. cinnamomi* (Davison, 1997; Burgess *et al*., 1999a). However, the compaction of clay in ponded riplines does not mean that underlying soil is waterlogged (Burgess *et al*., 1999b). The rehabilitated mine site area discussed here consisted of three components: (1) the field trial with clonal plants, (2) the seedling plot and (3) the two-year-old rehabilitated and revegetated area (Fig. 5.3 and Fig 5.22). Molecular analysis of samples taken from a dying *E. marginata* in the revegetated area a few metres from the dead seedlings indicated an isolate of *P. cinnamomi* different from that used to inoculate the clonal plants in the field trial (Dr. M. Dobrowolski, *pers. comm*). The *P. cinnamomi* isolate recovered from one of the dead non-inoculated clonal control plants was also not

the isolate used as inoculum (Dr. M. Dobrowolski, *pers. comm.)* and this indicates that the clonal plants used in the field trial were not totally resistant to the pathogen. While this may appear to contradict the low recovery from inoculated clonal plants, the two non-inoculated clonal plants which died during the trial were in a low-lying and ponded area and became infected when different environmental conditions prevailed to the conditions at the time of inoculation. Other unaffected clonal plants were higher on the soil gradient. The deaths of the seedlings and the non-inoculated clonal plants emphasizes the necessity of land preparation which avoids ponding. Future research could investigate interactions between environmental conditions, time of inoculation and disease development in different genotypes within a species, with an aim to develop clonal plants resistant to waterlogging and to *P. cinnamomi* infection.

The lesioned stems of the seedlings were later used in the preparation of an exudate solution to investigate the effect of exudates from infected stems on the growth of *P. cinnamomi* (Chapter 9), after *P. cinnamomi* was not recovered from some inoculated stems of the clonal plants with lesions characteristic of *P. cinnamomi* infection. This took the research into a new direction.

The effect of water status on the development of *P. cinnamomi* lesions has been observed in *E. marginata* (Tippett *et al*., 1987; Bunny *et al*., 1995) and in *E. sieberi* (Smith and Marks, 1986) in forest situations. All found an increase in lesion development in plants without water deficit. The current study was designed to investigate the development of disease in plants under different watering regimes in mine site conditions. Instead, the opportunity to make observations of the development of disease before and after unseasonally high summer rainfall was presented. As global weather patterns are altered, plant disease is likely to be of greater significance and modelling to provide accurate predictions of climatic conditions (Chakraborty *et al*., 1998) will benefit economic and environmental management strategies, including mine site rehabilitation.

5.4.5 *Conclusion*

This field trial clearly confirmed the importance of high moisture status to *P. cinnamomi*, not only for the development of disease *in planta* but also for its rapid advance through soil. However, it also demonstrated the vulnerability of an

experimental design reliant on weather conditions. In this trial, plants did not experience the extended drought conditions as planned. To investigate further the effect of drought on the development of disease caused by *P cinnamomi*, additional experiments were made in controlled conditions in the glasshouse (Chapters 6, 7 and 8).

Chapter 6

Development of disease in a clonal line of *Eucalyptus marginata* inoculated with, but resistant to, *Phytophthora cinnamomi*, when subjected to different watering regimes.

6.1 Introduction

The impact of disease caused by *Phytophthora cinnamomi* in the *Eucalyptus marginata* (jarrah) forest of Western Australia is well documented (Shearer and Tippett, 1989; Crombie and Bunny, 1994; Cahill, 1995). The pathogen infects not only this keystone species but targets a wide host range of understorey species. The susceptibility of indigenous flora to this exotic pathogen presents an additional challenge to rehabilitation work, after mining in a forest area is complete. The survival of inoculum, as chlamydospores in the soil, particularly at depth (Shearer and Shea, 1987), and in host tissue (Tippett *et al*., 1985) means that the potential for an outbreak of *P. cinnamomi* is a continual threat. The development of clonal lines of *E. marginata* plants, resistant to *P. cinnamomi* (Cahill *et al*., 1992; Cahill *et al*., 1993; McComb *et al*., 1994), which can be planted in rehabilitation sites, helps to counter this threat. Greater understanding of substrate moisture content as a factor in the development of disease can lead to more informed management strategies in mine site rehabilitation, e.g. it could impact on planting times and times of treatment with the fungicide, phosphite.

After unseasonal summer rainfall negated the droughted treatment in a field trial at a bauxite mine site (Chapter 5) it was decided to test the impact of water regimes on disease development in a series of trials in the glasshouse. Throughout these glasshouse experiments (Chapters 6, 7 and 8), the newly developed technique of inoculating plants with mycelial plugs, without wounding (Chapter 4), was employed. A previous trial (Chapter 3), showed that inoculated seedlings can die very quickly when the moisture content of the substrate is kept at container capacity for 7 days after inoculation. In this experiment, to test the impact of drought on infection and disease development, the clonal plants were exposed to drought immediately after inoculation. This simulated field conditions, commonly observed in Western Australia, where relatively high

rainfall coupled with warm temperatures in spring, providing conditions that are conducive to the reproduction of the pathogen, are normally followed by summer drought.

The aims of this trial were (1) to determine if drought could impede colonization by *P. cinnamomi* in the tissue of resistant *E. marginata* clones after inoculation at optimal moisture conditions, and (2) to determine if simulated summer rainfall after drought would induce an outbreak of *P. cinnamomi* in *E. marginata* plants in which the spread of the pathogen had been contained.

H_0: Drought and summer rainfall, as simulated in the glasshouse, have no effect on lesion development and colonization in stem tissue of clonal *Eucalyptus marginata*, selected for resistance to *Phytophthora cinnamomi*.

6.2 Methods

6.2.1 *Experimental Design*

The trial was a completely randomized block design and consisted of 6 treatments (3 watering regimes, with inoculated and non-inoculated plants) with 20 replicate plants per treatment (Table 6.1). Five replicates from each of the 6 treatments were randomly placed on each of 4 benches (blocks). Different watering regimes were imposed immediately after inoculation.

Table 6.1 Protocol for inoculating *E. marginata* clonal plants (1J30) with *P. cinnamomi* under 3 different watering regimes. Harvests were conducted 66 days (Harvest 1), 86 days (Harvest 2) and 108 days (Harvest 3) after inoculation.

Watering regime #	Inoculated	Number of plants harvested		
		Harvest 1	Harvest 2	Harvest 3
1	+	5	5	10
1	-	5	5	10
2	+	7	5	8
2	-	5	5	10
3	+	5	5	10
3	-	5	5	10

(+) = plants inoculated with mycelial plugs
(-) = control plants sham-inoculated with sterile agar plugs

Watering regime #, as illustrated in Figure 6.1, were:
(1) substrates of clonal plants were kept at container capacity (CC);
(2) clonal plants were droughted to wilting point (WP), then 10% of moisture loss from CC to WP was replaced and the substrate maintained at that level until harvest;
(3) as (2), but after 2 weeks at the droughted level, moisture content of substrate was restored to CC.

6.2.2 *Plant material and preparation*

Eighteen-month-old plants from a clonal line of *E. marginata* (1J30) from Alcoa World Alumina's Manjimup nursery were prepared for the glasshouse experiment. This clonal line was described as resistant to *P. cinnamomi* infection (McComb *et al*., 1990; Cahill *et al*., 1993 where it was described as 1-030). Four weeks prior to the start of the experiment, all plants were re-potted in free-draining 200mm polyvinyl pots, containing a commercial mix of sand, peat, sawdust and wood chips, with added micronutrients and a low-P slow release fertilizer (Richgro Product # SSM 1590, Richgro Pty. Ltd., Canningvale, WA. 6155). The plants were staked, tips pruned and basal branches removed to provide greater stem uniformity. Five grams of Isobutylidene diurea (IBDU), active ingredient 31% N, was applied to the substrate surface in each container 3 weeks prior to inoculation. They were hand-watered to container capacity twice daily for 3 weeks in an evaporatively-cooled glasshouse prior to the commencement of the experiment. For the duration of the experiment, maximum temperatures in the glasshouse ranged from 22.5^{o}C to 36.7^{o}C and minima from 13.2^{o}C to 24.7^{o}C. Heights and stem diameters were recorded at inoculation.

6.2.3 *Media*

An agar medium, selective for *Phytophthora*, NARPH (Appendix 3), was used for plating of stem segments (1) after re-passaging the pathogen through a host before the experiment, (2) after harvesting stems and (3) after leaching and replating of inoculated stems. A nutrient medium, V8 agar (Appendix 2a), was used in sham inoculations and to grow axenic cultures of *P. cinnamomi* as inoculum.

6.2.4 *Inoculum preparation*

Two jarrah seedlings were inoculated using a non-wounding technique as described in Chapter 4. The *P. cinnamomi* isolate used was MU 94-48 (mating type A_2) which had been assessed as highly virulent in jarrah (Hüberli, 1995). After three days, surface lesions were observed, the seedlings were harvested and segments of infected stem were plated onto NARPH. The outgrowth of *P. cinnamomi* mycelium from these segments was then aseptically sub-cultured onto V8 agar. This technique of passaging the pathogen through a host plant re-establishes the pathogen's aggressiveness, which can decrease after numerous *in vitro* subculturings (Jeffrey *et al*., 1962; Leach and Rich, 1969; Erwin, 1995). A pilot trial (Appendix 9) indicated faster *in vitro* growth of the re-passaged *P. cinnamomi* isolate MU 94-48. The resulting axenic culture of *P. cinnamomi* after re-passaging was again aseptically sub-cultured onto a medium of V8 agar (15 ml per 90 mm Petri dish) one week prior to inoculation of the clonal plants. A prepared template, with a grid of 1 cm x 1 cm squares, placed beneath the Petri dish allowed the medium to be cut accurately, with a sterile scalpel, into uniform square mycelial plugs.

6.2.5 *Inoculation*

The inoculation method used was as described in Chapter 4. Briefly, all plants were pre-treated by moistening the stem in an internodal area about 5 cm above the soil line where a periderm had formed. A sterile wad of wet cotton wool was applied to the stem and held in place with Parafilm™. After three days this was removed and, on those plants to be inoculated, a 1 cm x 1 cm mycelial plug was applied to the pre-moistened area. Another sterile wet cotton wool wad was placed over this plug and again held in place with Parafilm™ wrapped around the stem. Control plants were

treated in the same way but sham inoculated with sterile V8 agar plugs instead of the mycelial plugs. All wraps and plugs were removed after 14 days and the region of inoculation (ROI) where the plug had been placed was marked with a white correction pen (Mitsubishi Pencil Co. Ltd., Japan). Remaining inoculum was plated onto V8 agar in Petri dishes and incubated in the dark at 24±1^{o}C for 7 days to confirm its continuing viability after exposure to glasshouse conditions.

6.2.6 *Watering regimes*

Watering regimes (Fig. 6.1*)* were imposed immediately after inoculation. The substrates of one third of the plants were maintained at container capacity by hand-watering twice daily throughout the trial.

One third had water withheld from the day of inoculation until they reached wilting point (WP) after which the moisture content of the substrate was restored to the moisture content at WP plus 10% of the moisture loss from CC to WP. They were maintained at this droughted level, by daily weighing and restoration of the daily water loss, until harvest.

One third had water withheld from the day of inoculation until they reached WP after which the moisture content of the substrate was maintained at the droughted level of moisture content at WP+ 10% of the moisture loss (CC-WP) for 14 days. Moisture content was then restored to CC, simulating a summer rainfall event after drought. Substrate of plants was kept at CC until harvest. The first harvest was 21 days after restoration to CC.

Silver ducting tape was wrapped around the base of containers, covering drainage holes, to prevent leakage of water from the container while the moisture level of substrates of droughted plants was maintained. When substrates were restored to CC, the tape was removed and substrates kept close to CC in free draining pots.

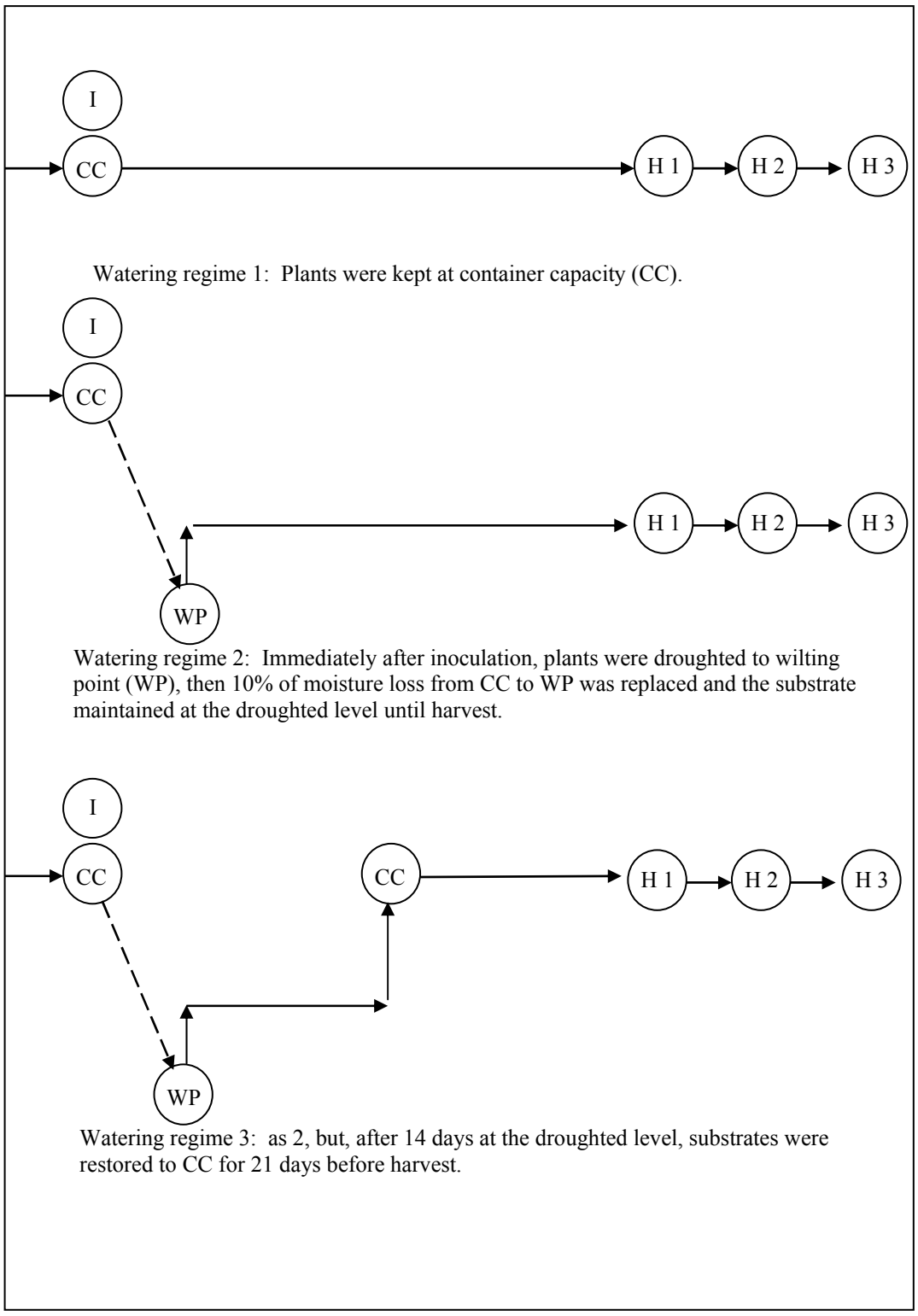


Figure 6.1 Schematic diagram of watering regimes in the glasshouse experiment with a clonal line (1J30) of *Eucalyptus marginata* plants. Harvest 1 (H1), Harvest 2 (H2) and Harvest 3 (H3) are 66, 86 and 108 days after inoculation, respectively.

6.2.7 *Monitoring water deficit and plant stress*

Moisture loss in all plants was monitored by daily weighing. All plants were monitored daily for any visible manifestation of infected tissue, lesions on the stem surface, and especially in droughted plants, for loss of turgidity in tips of new growth. This loss of turgidity was the observation used to determine WP. Weight of all replicates was recorded at the start of the experiment when all substrates were at CC (W_{CC}). The weight at WP (W_{WP}) was used in the calculation of the moisture level at which substrates of droughted plants were maintained (W_M) (Equation 6.1).

$$\text{Equation 6.1:} \quad W_M = W_{WP} + 10\%(W_{CC} - W_{WP})$$

Xylem pressure potential was measured when plants reached wilting point using a pressure chamber similar to the Scholander Pressure Bomb (Scholander *et al*., 1965) as described in Chapter 5.

Stomatal conductance of each plant was measured using a Delta ΔT AP4 cycling porometer (Delta T Devices Ltd., Cambridge, England.) The porometer was calibrated prior to each use. The youngest fully expanded leaf (YFEL) on each plant was marked with a thread and stomatal conductance of this leaf recorded on the day of inoculation and a further 6 times over the next 21 days. Measurements were discontinued after 21 days because of equipment failure.

6.2.8 *Mortality*

Plants which died, prior to the scheduled first harvest date, were harvested and plated onto the selective agar (6.2.3) and the results included in the Harvest 1 data.

6.2.9 *Harvests*

The 20 replicates in each treatment were to be harvested at 9, 12 and 14 weeks after inoculation - five plants from each treatment in the first and second harvests and 10 plants from each treatment in the third harvest (Table 6.1). At harvest, stems were cut at the soil line and side branches removed. Segments were marked in 1 cm increments, from 10 cms above the base of the region of inoculation (ROI) and down to

the ROI. Because no lesions were observed in these clonal plants, resistant to *P. cinnamomi*, an additional treatment to optimize the recovery of *P. cinnamomi* was introduced. This involved placing the stem segments in water for 5 days to remove the leachates prior to plating each segment onto selective agar. Briefly, at each harvest, the 1 cm segments were halved with a transverse cut and the lower half of each segment, wood and bark separated, was plated directly onto NARPH and the plates incubated at 24 ± 1^{o}C. A solution of sterile distilled water with added nutrients (5 ml L^{-1} cleared V8 juice) was warmed to 20^{o}C (pH 6.2). The upper halves of the stem segments were then added, and incubated in solution, at 24^{o}C in the dark for 5 days. The solution was changed daily to leach away stem exudates prior to the segments being plated onto NARPH. Plates were monitored for presence or absence of *P. cinnamomi* over 14 days.

At the third harvest, the above leaching process was modified by placing stem segments, after 5 days of leaching, under light at 24^{o}C for 48 hours, after which time the segments were plated onto NARPH. This modification was incorporated because the formation of *P. cinnamomi* sporangia have been observed after the exposure of mycelium to continuous light (Schoulties and Baker, 1974, cited by Zentmyer, 1980). This is also a step successfully used in zoospore preparation (Appendix 5). Though no sporangia were observed on stem segments after the light treatment, it was possible that microbial organisms in soil, and possibly present on the stem segments, could further stimulate the production of sporangia (Zentmyer, 1980), prior to plating. Plates were monitored as before.

6.2.10 *Statistical analysis*

Data were analysed using Statistica® for Windows Version 5 (StatSoft Inc., OK, USA) and Excel for Windows (Microsoft Corporation, USA), Microsoft Excel Analysis ToolPak (GreyMatter International, Inc., Cambridge, MA 02141). Prior to parametric analyses, data were screened for basic assumptions of parametric tests including normal distribution, equality of variances and non-correlation of means and variances. In cases where data did not conform to assumptions, log transformations were used to correct the deviations. The use of such transformations is noted in the relevant results. Where data were log-transformed, means are presented graphically for

non-transformed data. After comparison of data between or within plant groups (Table 6.1), where an ANOVA resulted in a P-value of < 0.05, data were considered significantly different.

6.3 Results

6.3.1 *Plant growth and glasshouse temperatures*

Mean height of all plants at the beginning of the trial (*n* =120) was 97.28 ± SE 1.27 cm. Mean stem diameter (*n* = 120) was 0.66 ± SE 0.09 cm. One way ANOVAs showed no significant (*df* 5,114; P>0.05) difference in height (Table 6.2) or in stem diameter of plants between treatments at the start of the experiment. The experiment was conducted between April and July, 2000 when temperatures in the glasshouse ranged from 13.2°C to 36.7°C (Table 6.3).

Table 6.2 Heights of *E. marginata* plants at inoculation (prior to imposition of watering regimes).

Treatment	Watering regime	Inoculated or Control	Replicates *n* =	Mean height and SE (cm)
1	1	Inoculated	20	92.20 ± 2.26
2	1	Control	20	97.20 ± 3.65
3	2	Inoculated	20	95.10 ± 2.98
4	2	Control	20	99.75 ± 3.48
5	3	Inoculated	20	103.45 ± 1.99
6	3	Control	20	96.00 ± 3.59

Watering regimes are described in Figure 6.1. SE = standard error of the mean.
cm = centimetres.

Table 6.3 Temperatures in the glasshouse

Prior to	Dates	Maxima (°C)	Minima (°C)
Harvest 1	Apr 10 – June 15	22.5 to 36.7	13.3 to 24.7
Harvest 2	June 16 – July 4	25.8 to 30.9	13.8 to 21.5
Harvest 3	July 5 – July 27	23.9 to 31.4	13.2 to 19.0

6.3.2 *Wilting Point*

Droughted plants (n = 80) took from 8 to 28 days to reach WP (Table 6.4). There was no significant (*df* 1,78; P >0.05) difference between inoculated and control plants. Median value (days to WP) of both inoculated and control plants was 13.

Table 6.4 Days taken to reach wilting point (WP) by *E. marginata* plants of a clonal line (1J30) resistant to *P. cinnamomi*, after inoculation.

Watering regime	Inoculated or Controls	Days to WP Mean and SE	Range of days to WP	Replicates n =
2 and 3	Inoculated	15.00 ± 0.79	8 - 28	40
2 and 3	Controls	14.28 ± 0.68	8 - 25	40

Watering regimes 2 and 3 are described in Fig. 6.1. SE = standard error of the mean.

Xylem pressure potential values, at wilting point, ranged from –1.1 MPa to –3.6 MPa in control plants and from –1.94 MPa to –3.52 MPa in inoculated plants. Therre was no significant (*df* 1,58; P>0.05) difference between the XPP of droughted, inoculated plants at WP and droughted, control plants at WP.

6.3.3 *Stomatal conductance*

The trend was for values of stomatal conductance readings for the *E. marginata* plants of a clonal line (1J30) resistant to *P. cinnamomi* to decrease with time after inoculation. Mean stomatal conductance of all droughted treatments decreased after inoculation, but after an initial decrease, that of plants kept at CC throughout the trial increased sharply 11 days after inoculation (Fig. 6.2). Equipment failure prevented continued monitoring of stomatal conductance.

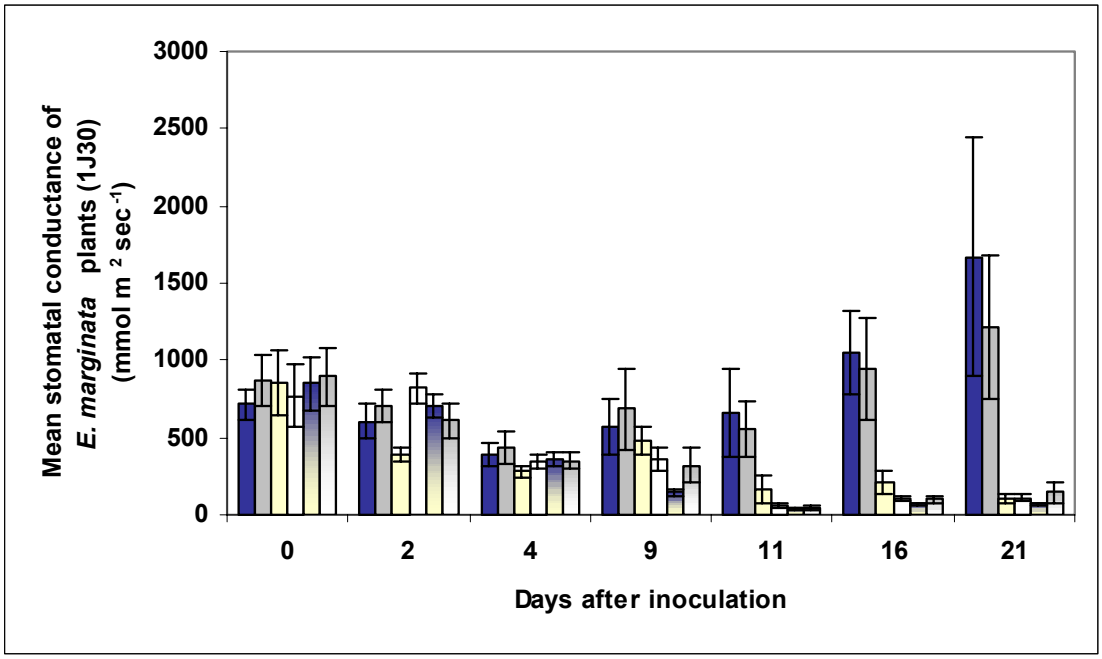


Figure 6.2 Mean stomatal conductance (mmol m^2 sec^{-1}), of *E. marginata* (1J30) leaves. Seven readings were taken of the six treatments (key below).
Bars represent the standard error of the mean.

Watering regime 1 Inoculated seedlings kept at container capacity (CC) throughout the trial.

Watering regime 1 Control (sham-inoculated) seedlings kept at container capacity (CC).

Watering regime 2 Inoculated seedlings droughted to wilting point (WP) then maintained until harvest at a droughted level, with 10% of substrate moisture lost from CC to WP restored.

Watering regime 2 Control (sham-inoculated) seedlings droughted as in Watering regime 2.

Watering regime 3 Inoculated seedlings droughted as in 2, but restored to CC after 14 days of droughted conditions.

Watering regime 3 Control (sham-inoculated) seedlings droughted then restored to CC as in Watering regime 3.

6.3.4 *Mortality*

There was one death, prior to the first harvest, of an inoculated plant kept at CC from which *P. cinnamomi* was recovered. There were 7 plant deaths in droughted treatments prior to the first harvest. Of these, 5 were inoculated plants maintained at the droughted level, and early deaths occurred at 3, 7, 9, 10 and 20 days after reaching WP. Two were control plants which died 6 days after reaching WP and before restoration to CC. *P. cinnamomi* was recovered from only 2 of the dead inoculated plants and from neither of the control plants.

6.3.5 *Lesions and colonization*

No lesions were observed on any stems during this trial. Of the 60 clonal *E. marginata* plants (1J30), inoculated with *P. cinnamomi*, only 13 were colonized by the pathogen. Six of these had been kept at CC throughout the trial (watering regime 1). The greatest extent of colonization was seen in 2 of the plants that died prior to Harvest 1. *P. cinnamomi* was recovered from one plant which had been kept at CC and from one plant maintained at the droughted level, from the ROI to 6 cm and 8 cm above the ROI, respectively. Any other colonization, in plants in any treatment, were from the ROI to 4 cm or < 4 cm above the ROI.

There was a decrease in the extent of colonization in each successive harvest in watering regimes 1 and 2 (Fig 6.3). Few replicates contributed to the data, but a two-way ANOVA of log-transformed data showed that neither the time of harvest nor the treatment (watering regime) had a significant (*df* 2,51; $P>0.05$) effect on the total colonization of stems.

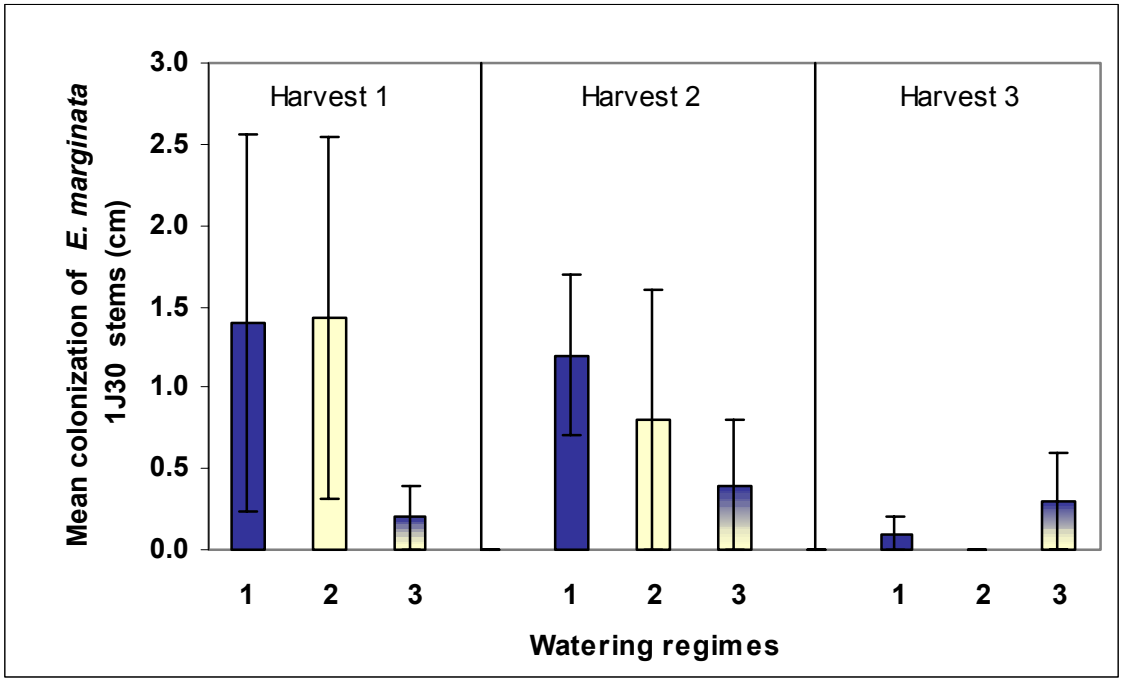


Figure 6.3 Mean extent of colonization of tissue in stems of a clonal line (1J30) of *E. marginata* plants, resistant to *P. cinnamomi*, subjected to 3 watering regimes. Bars represent the standard error of the mean.

Watering regime 1 Inoculated plants kept at container capacity (CC) throughout the trial.

Watering regime 2 Inoculated plants droughted to wilting point (WP) then maintained until harvest at a droughted level, with 10% of moisture lost from CC to WP replaced.

Watering regime 3 Inoculated plants droughted as in 2, but restored to CC after 14 days of droughted conditions

6.3.6 *Recovery*

Over the 3 harvests, most recoveries of *P. cinnamomi* were made from plants kept at container capacity. There were 8 recoveries made from direct plating of the 60

inoculated stems. Five additional recoveries were made after leaching, in Harvest 1 (Table 6.5). No recovery was made from stems of control plants

Table 6.5 Number of replicates of *E. marginata* (clonal line 1J30) stems from which *P. cinnamomi* was recovered with direct plating and number of additional recoveries after leaching. There were 5 – 10 plants in each treatment in each harvest (Table 6.1).

	Harvest 1		Harvest 2		Harvest 3		
Watering regime #	direct plating	after leaching	direct plating	after leaching	direct plating	after leaching	Totals
1	1	+1	3	0	1	0	6
2	0	+3	1	0	0	0	4
3	0	+1	1	0	1	0	3
Total:	1	+5	5	0	2	0	13

Watering regimes are as described in Figure 6.1. Harvests 1, 2 and 3 were 66, 86 and 108 days, respectively, after inoculation.

6.4 Discussion

6.4.1 *Factors influencing recovery of* P. cinnamomi

Water status played a role in the number of recoveries of *P. cinnamomi* made. Most recoveries of *P. cinnamomi* from these resistant clonal plants were from those kept at container capacity (CC) throughout the trial. Over the three harvests, 13 recoveries of *P. cinnamomi* were made, of which 6 were from the 20 plants kept at CC. Of the 40 plants droughted after inoculation, 7 recoveries were made, which suggests that droughted plants were less easily colonized by the pathogen.

Five of the six *P. cinnamomi* recoveries made in Harvest 1 occurred after leaching the stem segments of inoculated plants from which there was no initial recovery. Modifications made to the leaching method, prior to plating onto selective agar and incubation in the dark at 24±1^{o}C, included the addition of nutrients and an adjustment of temperature and pH of the leaching solution to conditions favourable to growth of the *P. cinnamomi* (Section 6.2.9). In Chapter 5 and Chapter 9, leaching of segments had been at fluctuating room temperatures, but maintenance of temperature by incubating segments, in the amended leaching solution, in the dark at 24±1^{o}, would have provided better conditions for the growth of any hyphae in the stem tissue. Exudates

from infected stems of *E. marginata* have been shown to lower the pH of the solution and to inhibit the growth of *P. cinnamomi* mycelium (Chapter 9). Leaching away these exudates and replacing them with nutrient solution with pH adjusted to 6.2 and temperature to 20^{o}C in Harvest 1, substantially improved the rate of recovery of the pathogen from the resistant clonal plants. This success was not repeated in Harvest 2 or Harvest 3, suggesting that the time elapsed from infection to harvest, particularly in resistant hosts, is another important factor in recovery. Time may allow the resistant host to 'wall off' and contain the *P. cinnamomi*, with lignification at the barriers between necrotic and healthy tissue, inducing dormancy or possible death of the pathogen.

After the lack of further recovery at Harvest 2, the stem segments from Harvest 3 were placed in the leaching solution under light at 24±2^{o}C for 48 hours to induce the formation of sporangia. This step, with hyphae in soil filtrate, results in successful formation of sporangia and subsequent production of zoospores (Appendix 5). The lack of success in the leaching solution may have been due to an abundance of nutrients and/or insufficient microbial action. Sporangia form when nutrients are lacking and soil microbes stimulate their formation (Zentmyer, 1980). The leaching of exudates from infected stem segments has proved to be an effective process previously (O'Gara, 1998; Hüberli *et al*., 2001; and Chapter 9) to yield further recoveries of *P. cinnamomi* with hyphal growth no longer inhibited. In the current study, the method was sufficient in Harvest 1 but not thereafter. This observation reinforces the concept that the length of time elapsed from infection to harvest is a determining factor in the recovery of *P. cinnamomi* from a resistant host.

Total colonization of stem tissue also decreased over time in watering regime 1 (plants kept at CC) and watering regime 2 (plants maintained at the droughted level after reaching WP), with a slight increase in watering regime 3 (plants restored to CC after droughting) (Fig. 6.3). The increase in colonization of plants when restored to CC compared to that of plants kept at CC may be because constant, more stable, conditions allowed the resistant host, kept at CC, to contain the *P. cinnamomi*, while the sudden uptake of water with the increased supply of nutrients, after the substrate was restored to CC, may have allowed an outbreak of the disease in the other plants. However, with the data supplied by only a small proportion of recoveries, the results are inconclusive.

Other researchers have found that recovery from jarrah diminishes with time in forest conditions (Davison *et al*., 1994) and in rehabilitated bauxite mine site conditions (O'Gara, 1998). However, it is unclear whether this happens because the pathogen is dying or becoming dormant. The continued synthesis of fungitoxic or fungistatic phenolic compounds by the host may cause death or trigger the implementation of a survival strategy by the pathogen. Further research is needed to clarify the changing status of the pathogen in diseased tissue.

Temperatures in the glasshouse (Table 6.3) were conducive to the growth of *P. cinnamomi* (Phillips and Weste, 1985) and to the isolate used in this experiment (Hüberli *et al*., 1997), so the ambient temperatures would not have inhibited the infection process. The inoculation method was successful with seedlings of *E. marginata* in the glasshouse (Chapter 4) and with clonal plants of *E. marginata* (77C40), resistant to *P. cinnamomi* (M. Stukely, *pers. comm*.) in the field (Chapter 5). The immediate imposition of water deficit at the time of inoculation, rather than 7 days after inoculation (as in Chapter 3), may have hindered pathogenesis in the droughted plants. However, there is also a great difference in the proportion of infected clonal plants kept at CC in this experiment compared to that of seedlings in Chapter 3. Both inoculation methods used - zoospore inoculation in Chapter 3 and mycelial plug inoculation in this experiment - are non-wounding techniques. It has been suggested that potential hosts recognize zoospores differently from invasive hyphae (Cahill *et al*., 1989). However, the disproportionate results between the seedlings in Chapter 3 and the resistant clonal plants in this chapter, when the mycelial inoculum was confirmed as an actively growing isolate of *P. cinnamomi* (Section 6.2.5), indicate a high level of resistance in this clonal line of *E. marginata* (1J30) compared to the seedlings. This line of clonal plants has been shown to be a very resistant *to P. cinnamomi* infection. No lesions were observed on stems in any treatment in this trial. The limited extent of colonization in stems from which *P. cinnamomi* was recovered in this resistant clonal line (1J30) of *E. marginata* endorses the findings of Cahill *et al*. (1993) where lesions on roots of the resistant clonal *E. marginata* plants infected with *P. cinnamomi*, were significantly less than on roots of seedlings. Hüberli *et al*. (1997) also found stem-inoculated 1J30 to be the most robust, longest surviving, clonal line at temperatures up to 20^{o}C.

6.4.2 *Indications of stress in the development of disease*

There was no significant difference in the number of days taken to reach wilting point (WP) and no significant difference in xylem pressure potential (XPP) at WP between inoculated plants and control plants. Tippett *et al*. (1987) found that growth of *P. cinnamomi* was inhibited in *E. marginata* at –1.5 MPa pre-dawn XPP. Readings of all but one of the droughted plants at WP in this trial, both inoculated and control plants, were lower than –1.5 MPa. Except for one droughted control plant, mid-morning readings were from -1.8 MPa to -3.6 MPa. Though not an entirely valid comparison, the readings in this trial were well below the –1.5 MPa point of inhibition and do indicate severe stress. The restoration of 10% of lost moisture to the plants' growing medium would have reduced the water deficit and altered the xylem pressure potential (XPP) to a less negative reading. Because the sampling of leaves or branchlets, for pressure chamber readings of XPP, is a destructive and wounding process which would have implications of plant defence reactions in this trial, no further XPP readings were taken. Growth of *P. cinnamomi* may have been impeded by the low potential in these droughted stems at WP. It is important that future research examines what happens to the pathogen with small changes in water status from a droughted condition.

The high mortality prior to harvest indicated severe stress in plants. After recovery of *P. cinnamomi* from one plant kept at CC, the cause of death can be assigned to the pathogen. However, since 2 non-inoculated, droughted plants also died prior to harvest, it is likely that the level of water deficit was too great for these clonal plants to withstand. Consequently, of the 5 droughted and inoculated plants which died, it is difficult to ascertain which stress contributed most to their deaths. While *P. cinnamomi* was recovered from only 2 of these 5 plants, either stressor (drought or disease) may have been responsible for the deaths. Control plants succumbed to drought, and it is possible that inoculated plants, and the colonizing pathogen in the plant tissue, did also.

Stomatal conductance was variable both in non-inoculated control plants kept at CC and in inoculated, droughted plants (Fig. 6.2). Stomatal conductance can be influenced both by water content of plant tissue and by disease. The interaction of both factors in this trial may have contributed to the apparently inconsistent readings. Diseased *Eucalyptus* spp. can initially register a higher rate of stomatal conductance

which, with time, declines as the host adapts to the disease (Crombie and Milburn, 1988). The highest readings in this trial were of plants kept at CC, 16-21 days after inoculation (Fig. 6.2). This is also the watering regime which had the highest incidence of disease (Table 6.5 and Fig. 6.3). Lower stomatal conductance of plants in the droughted treatments could indicate a less pathogenic effect coupled with the stress of water deficit. In a glasshouse trial, Stoneman *et al*. (1994) recorded lower stomatal conductance in droughted seedlings of *E. marginata*, compared to watered and rewatered seedlings. Measuring stomatal resistance (inverse of stomatal conductance) of *Eucalyptus* spp. in the field, Landsberg and Wylie (1983) found that readings for trees affected by dieback (but with minimal recovery of *Phytophthora*) were lower than those for healthy control trees. In the current study, the trend over time (0-21 days after inoculation) was for a decrease in stomatal conductance, with the interim exception of plants in Treatment 1 (kept at CC) as noted previously. Continued observations could not be made because the equipment failed. Though means are of readings of the same treatment, within that treatment plants are at different stages of droughting prior to and after WP, because they all took a different length of time to reach WP. This may also explain apparent inconsistencies. All plants in this trial were resistant to *P. cinnamomi*. Is drought stress more pronounced in resistant plants than disease stress? Interpretation of readings taken from drought-stressed and disease-stressed *E. marginata* plants resistant to *P. cinnamomi* can be considered with comparable readings from plants susceptible to *P. cinnamomi* and from seedlings in Chapter 8.

6.4.3 *Conclusion*

The paucity of infected stems (13 of 60 inoculated plants) and the limited extent of colonization (none > 8 cms above the ROI) is indicative of the resistance of this clonal line (1J30) of *E. marginata* plants to *P. cinnamomi*, but provides insufficient data with which to gauge the effects of the different watering regimes on the progression of the disease. The hypothesis will be tested again, using a clonal line of *E. marginata*, 11J402 (Cahill *et al*., 1993), susceptible to *P. cinnamomi* (Chapter 7), and also using both resistant and susceptible clonal lines and seedlings of *E. marginata* (Chapter 8).

Chapter 7

Development of disease in a clonal line of *Eucalyptus marginata* plants, susceptible to and inoculated with *Phytophthora cinnamomi*, when subjected to different watering regimes.

7.1 Introduction

In the current experiment, the methodology of Chapter 6 was repeated to retest the hypothesis that water deficit has a limiting effect on disease development, but a *E. marginata* clone (11J402), susceptible to *P. cinnamomi* (McComb *et al*., 1990; Cahill *et al*., 1993) was substituted for the resistant clone (1J30) (M. Stukely *pers.comm*). The same watering regimes were imposed and the timing of inoculation was the same. However, the current experiment was conducted in the spring, 6 months after the experiment described in Chapter 6, and temperatures in the glasshouse were higher. The susceptible plants were older, but of a similar height and stem diameter at the start of the experiment to the resistant clones. In Chapter 6 there were three harvests, but only one in the current experiment, which approximated the time of the first harvest in Chapter 6.

The aim of this experiment was to determine to what extent (1) drought (water deficit) and (2) simulated summer rainfall (the restoration of substrate moisture to container capacity) are factors in disease development in clonal *E. marginata* plants, designated susceptible to *P. cinnamomi*.

The null hypothesis, H_0 : Watering regimes will have no effect on the development of disease caused by *P. cinnamomi* in *E. marginata* plants of a clonal line susceptible to the pathogen.

7.2 Methods

7.2.1 *Experimental design*

The trial was a completely randomized block design and consisted of 6 treatments with 10 replicate plants per treatment (Table 7.1) which were subjected to different watering regimes immediately after inoculation. Plants were harvested 63 days after inoculation.

Table 7.1 Protocol for inoculating susceptible *E. marginata* clonal plants (11J402) with *P. cinnamomi* under 3 different watering regimes.

Watering regime	Inoculated	Number of replicates
1	+	10
1	-	10
2	+	10
2	-	10
3	+	10
3	-	10

(+) = plants inoculated with mycelial plugs
(-) = control plants sham-inoculated with sterile agar plugs
Watering regimes as illustrated in Figure 6.1 were:
(1) substrate of clonal plants was kept at container capacity (CC);
(2) clonal plants were droughted to wilting point (WP), then 10% of moisture loss from CC to WP was replaced and the substrate maintained at that level until harvest;
(3) as (2), but after 2 weeks at the droughted level, moisture content of substrate was restored to CC.

7.2.2 *Plant material and growing conditions*

Twenty-month-old *E. marginata* clonal plants (11J402), susceptible to *P. cinnamomi* (McComb *et al*., 1990), were provided by the Marrinup nursery of Alcoa World Alumina Australia. Plants of comparable height with naturally formed periderm extending more than 10 cm from the soil line were selected. They were potted in 200 mm free-draining vinyl pots filled with a container substrate specifically for native plants (Richgro mix #SSM 1590, Appendix 1b). Fertilizer (5g Osmocote® Low P – Scotts Australia Pty.Ltd., Cecil Avenue, Castle Hill, NSW Australia 2154) and 2g of IBDU (Isobutylidene diurea), active ingredient 31% N,) was added to the substrate surface in each container. The plants were acclimatised in the glasshouse for 3 weeks prior to the trial and watered twice daily to container capacity (CC). Temperatures in the

glasshouse were recorded daily. Heights and stem widths of all plants were recorded at the start of the experiment and the heights recorded again at harvest. Relative plant growth was calculated (Equation 7.1).

Equation 7.1: Relative growth = $\frac{\text{(Height 2 – Height 1) / Days to death or harvest}}{\text{Height 1}}$

7.2.3 *Inoculum preparation*

Mycelial plugs, colonized by *P. cinnamomi*, were prepared as described in Chapter 6 (Sections 6.2.3 and 6.2.4).

7.2.4 *Inoculation*

Pre-treatment, inoculation of plants and post-inoculation monitoring were as described in Chapter 6 (Sections 6.2.5 and 6.2.7). Wet cotton wool was applied over the plug of mycelial agar at inoculation. The remaining inoculum was returned to the laboratory, plated onto V8 agar and incubated at 24±1^{o}C, to confirm its viability after exposure to glasshouse conditions.

7.2.5 *Watering regimes*

Three different watering regimes, as described in Chapter 6 (Section 6.2.6 or Figure 6.1) were imposed (Table 7.1) immediately after inoculation. When the cotton wool and Parafilm™ wrap were removed 14 days after inoculation, the cotton wool still retained some moisture. Fourteen days after the first 10 droughted plants reached wilting point (WP), they were restored to, and kept at CC until harvest. The remainder of the droughted plants were maintained at WP + 10%(CC-WP) by watering to weight daily. Silver ducting tape was used to cover the drainage holes of the containers to prevent water loss during the droughted treatments. When the substrates of plants were restored to CC, tapes were removed and the substrates were watered to CC.

7.2.6 *Monitoring water deficit and plant stress*

The number of days to WP and the xylem pressure potential (XPP) of droughted plants at WP were recorded, using a pressure chamber similar to that described by Scholander *et al*. (1965).

Stomatal conductance was measured with a portable gas-exchange measurement system (PP Systems CIRAS-1 Combined Infra-Red Gas Analysis System) with a standard leaf cuvette (PP Systems CIRAS-1 Parkinson Standard Leaf Cuvette) at three critical points in the trial. These were:

(1) 10 days after inoculation (by which time disease symptoms and drought stress were evident).
(2) 23 days after inoculation (2 days after the first 10 droughted replicates to reach WP had been restored to CC), and
(3) at harvest, 63 days post inoculation, at which time all watering regimes had been imposed and maintained for >21 days.

Daily moisture loss from each container was recorded and daily observations made of the turgidity of tips of new growth prior to WP, of visible surface lesions and of general plant health.

7.2.7 *Mortality*

Any plants which died prior to the nominated harvest date, 63 days post inoculation, were harvested. Stem segments were plated onto NARPH selective agar as described in Chapter 6.2.9.

7.2.8 *Harvest*

All surviving plants were harvested 63 days after inoculation. The plants were treated as described in Chapter 6.2.9. Lesions on stems were measured and heights of all plants were recorded, at death or at harvest.

7.2.9 *Statistical analysis*

Data were analysed using Statistica® for Windows Version 5 (StatSoft Inc., OK, USA) and Excel for Windows (Microsoft Corporation, USA), Microsoft Excel Analysis ToolPak (GreyMatter International, Inc., Cambridge, MA 02141). Prior to parametric analyses, data were screened for basic assumptions of parametric tests including normal distribution, equality of variances and non-correlation of means and variances. In cases where data did not conform to assumptions, log transformations were used to correct the deviations. The use of such transformations is noted in the relevant results. Where data were log-transformed, means are presented graphically for non-transformed data. After comparison of data between or within plant groups (Table 7.1), where an ANOVA resulted in a P-value of < 0.05, data were considered significantly different.

7.3 Results

7.3.1 *Plant growth and glasshouse temperatures*

Mean height of all plants at the start of the experiment was 70.93 ± SE 1.50 cm. There was no significant (*df* = 5, 54; P>0.05) difference in the heights of plants between treatments. Mean stem width at the start of the experiment was 0.68 ± SE 0.01 cm. Most plant growth was in the non-inoculated plants kept at container capacity (CC) throughout the trial. A one-way ANOVA showed a significant (*df* 5,54; P=0.003) difference in relative plant growth (Equation 7.1) between treatments (Fig. 7.1).

Maximum daily temperatures in the glasshouse ranged from 27.2^{o}C to 43.3^{o}C and minima from 14.9^{o}C to 28.4 ^{o}C. Maximum temperature on the day of inoculation was 41.9 ^{o}C and on the day of harvest was 39.5 ^{o}C .

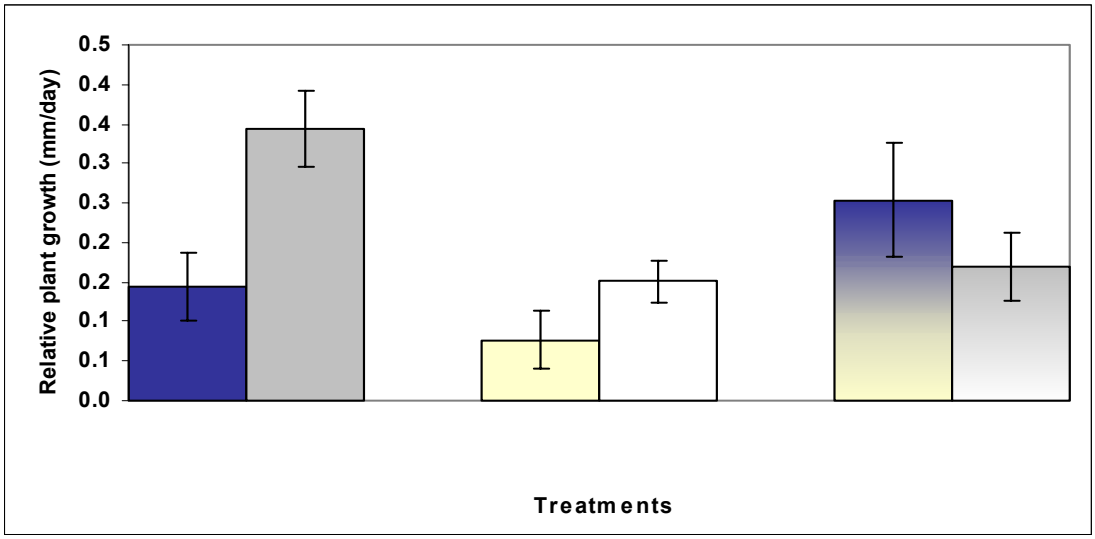


Figure 7.1 Growth of plants in all treatments, relative to height at the start of the experiment. Bars represent the standard error of the mean.

Watering regime 1 Inoculated plants kept at container capacity (CC) throughout the trial.

Watering regime 1 Control (non-inoculated) plants kept at container capacity (CC).

Watering regime 2 Inoculated plants droughted to wilting point (WP) then maintained until harvest at a droughted level, with 10% of moisture lost from CC to WP replaced.

Watering regime 2 Control (non-inoculated) plants droughted as in 2 (above).

Watering regime 3 Inoculated plants droughted as in 2, but restored to CC after 14 days of droughting.

Watering regime 3 Control (non-inoculated) plants droughted then restored to CC, as in 3 (above).

7.3.2 *Wilting Point*

Seven days after inoculation 15 plants reached WP (5 inoculated plants and 10 control plants). All droughted, inoculated plants (n = 20) had reached WP by 14 days and all droughted control plants (n = 20) by 18 days after inoculation (Fig. 7.2).

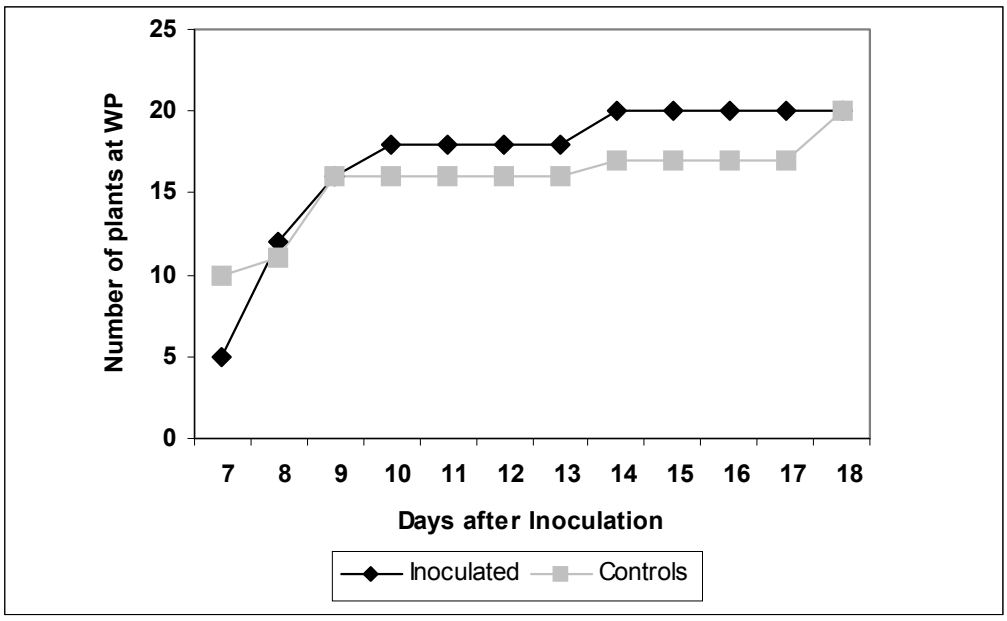


Figure 7.2 Time to wilting point (WP) for *E. marginata* plants of a clonal line (11J402), susceptible to and inoculated with *P. cinnamomi* (black) and non-inoculated control plants (grey).

Mean XPP for inoculated, droughted plants at WP was –2.75 ± SE 0.12 MPa, and was significantly (P = 0.011) lower than the mean for control plants which was –2.33 ± 0.10 MPa.

7.3.3 *Stomatal conductance*

Stomatal conductance tended to increase over time (0-63 days after inoculation). The mean stomatal conductance of all droughted treatments increased, but the values of inoculated plants kept at CC and non-inoculated plants kept at CC rose sharply before decreasing (Fig. 7.3).

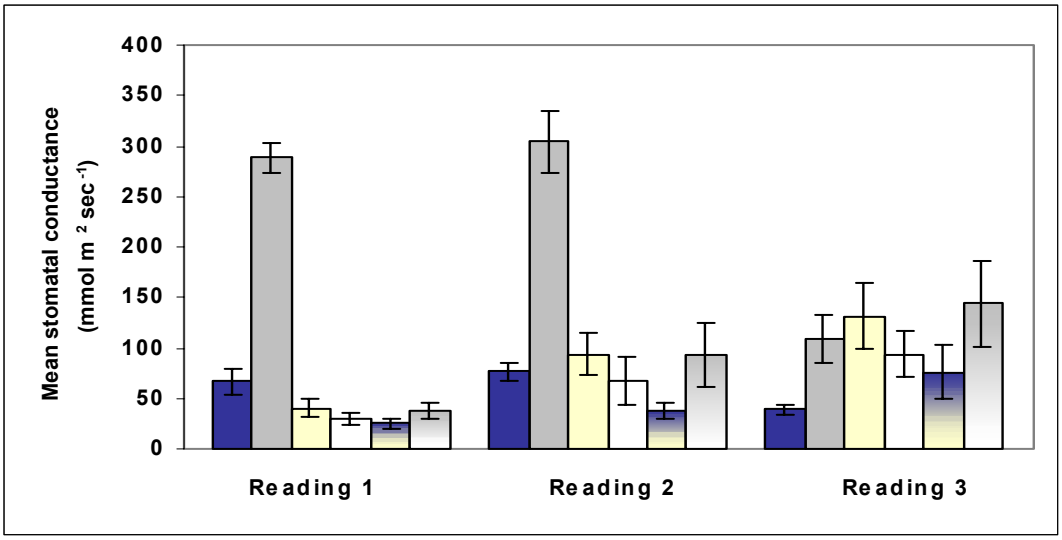


Figure 7.3 Stomatal conductance readings of leaves of a clonal line (11J402) of *E. marginata* plants, susceptible to *P. cinnamomi*.

Watering regime 1 Inoculated seedlings kept at container capacity (CC) throughout the trial.

Watering regime 1 Control (sham-inoculated) seedlings kept at container capacity (CC).

Watering regime 2 Inoculated seedlings droughted to wilting point (WP) then maintained until harvest at a droughted level, with 10% of substrate moisture lost from CC to WP restored.

Watering regime 2 Control (sham-inoculated) seedlings droughted as in Watering regime 2.

Watering regime 3 Inoculated seedlings droughted as in 2, but restored to CC after 14 days of droughted conditions.

Watering regime 3 Control (sham-inoculated) seedlings droughted then restored to CC as in Watering regime 3.

7.3.4 *Mortality*

The first 3 deaths occurred 13 days after inoculation in the droughted, inoculated plants. On day 14, an inoculated plant kept at CC, died. No further deaths were recorded until 24 days after inoculation when there were 5 deaths in droughted, inoculated plants (Table 7.2). Another inoculated plant kept at CC and two non-inoculated control plants, droughted then restored to CC were severely wilted but survived until harvest. No non-inoculated plants died.

Table 7.2 Mortality rate of a clonal line (11J402) of *E. marginata* plants, susceptible to and inoculated with *P. cinnamomi*.

Watering regime	Replicates inoculated *n* =	Deaths at 13 days	Deaths at 14 days	Deaths at 24 days	Total deaths prior to Harvest	Harvest *n* =	Total recovery of *P.cinnamomi* *n* =
1	10		1 (1)		1	9 (5)	6
2	10	3 (2)		2 (0)	5	5 (0)	2
3	10			3 (0)	3	7 (3)	3

Numbers in brackets denote number of replicates from which *P. cinnamomi* was recovered. Watering regimes 1, 2 and 3 were as described in Figure 6.1.

7.3.5 *Lesions and colonization*

Stem lesions were not visible until 14 days after inoculation when the pathogen had colonized the green stem area above the periderm. Lesions were seen only on the stems of 3 plants which were kept at CC throughout the trial. The rate of growth of these 3 lesions was recorded daily and their development was greatest in the first 2 weeks (Fig. 7.4). The longest stem lesion at harvest was 24 cm. A 3.5 cm lesion also developed along a branch of one of these plants. Plant tissue beyond the lesion front was also colonized. No surface lesions were seen on any other plants in any treatment. Inoculated stems in all watering regimes were colonized above and below the ROI. For comparison with results of other experiments (Chapters 3, 4, 5, 6 and 8), only the lesions and colonization at and above the ROI in the current study have been presented (Fig. 7.5).

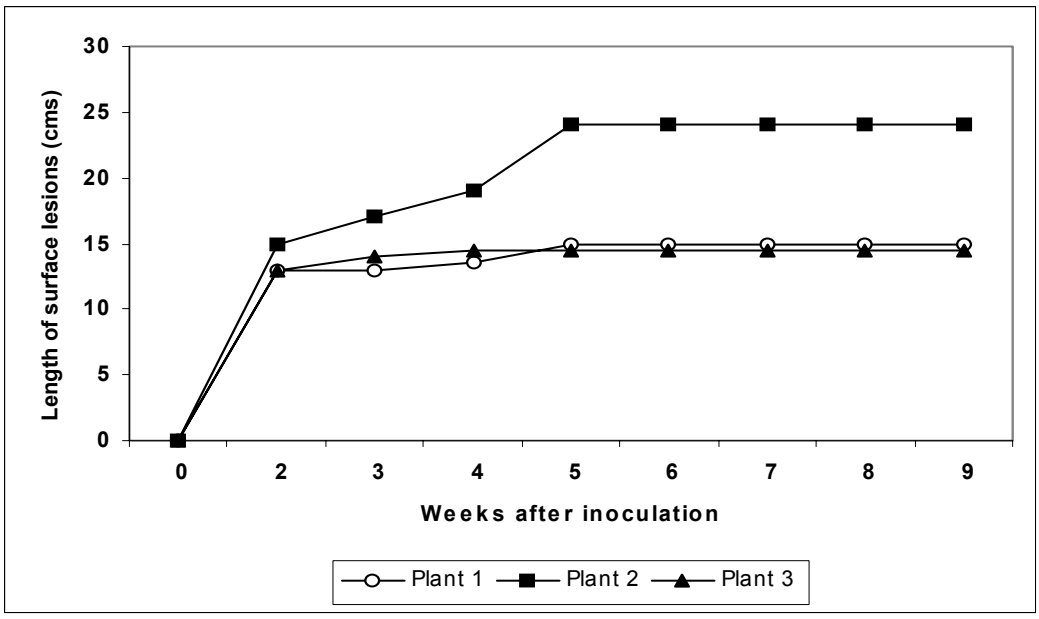


Figure 7.4 Development of the surface lesions visible only on stems of three inoculated *E. marginata* plants of a clonal line (11J402) kept at container capacity.

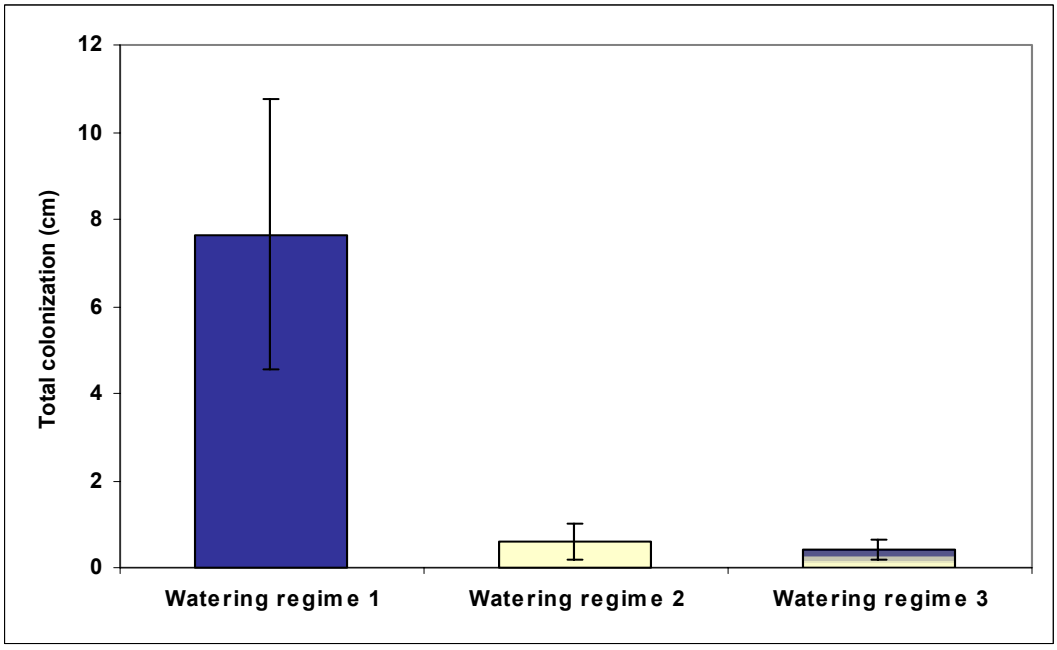


Figure 7.5 Total colonization of a clonal line (11J402) of *E. marginata* stems inoculated with and susceptible to *P. cinnamomi* in the 3 different watering regimes described in Table 7.1. Bars represent the standard error of the mean.

7.3.6 *Recovery of* P. cinnamomi

P. cinnamomi was recovered from 6, 2 and 3 plants in watering regimes 1, 2 and 3, respectively (n = 10 in all treatments) (Table 7.2). No *P. cinnamomi* was recovered from any control plant. All the inoculum plugs tested for viability immediately after inoculation of plants (Section 7.2.4) tested positive for *P. cinnamomi*. Intense staining of the NARPH selective agar by exudates from the stem segments of only one plant maintained at droughted level was noted. No further recoveries of *P. cinnamomi* were made after leaching of stem segments as described in Chapter 6 (Section 6.2.9).

7.4 Discussion

7.4.1 *Factors influencing the development of disease caused by* P. cinnamomi *in the clonal line (11J402) of susceptible* E. marginata *plants.*

Most colonization of inoculated stems was in plants that were kept at container capacity (CC) throughout the trial. This confirms the results of Chapters 3 (Harvest 2) and Chapter 6 that droughted *E. marginata* plants are less likely to be colonized by *P. cinnamomi* than those at CC. This has now been shown with seedlings of *E. marginata* (Chapter 3), clonal plants (1J30) resistant to *P. cinnamomi* (Chapter 6) and clonal plants (11J402) susceptible to *P. cinnamomi* (Chapter 7). Water status is a major factor in the development of the disease caused by *P. cinnamomi* in *E. marginata*.

Most recoveries of *P. cinnamomi* were also made from plants that were kept at container capacity. Overall, the proportion of recoveries made, 11 from 30 inoculated clonal plants (11J402), susceptible to *P. cinnamomi*, was low (36.7%). This is a higher proportion than was recovered from the resistant *E. marginata* (1J30), where *P. cinnamomi* could only be recovered from 13 of the 60 inoculated plants (21.7%) (Chapter 6). Further leaching of the stem segments, from which recovery was not made with direct plating, was not successful in this trial, though it had been in the previous trial (Chapter 6) when 5 additional recoveries were made after Harvest 1. Harvest 1 (Chapter 6) and Harvest (current study) were after 66 days and 63 days, respectively. No recoveries were made after leaching from later harvests in Chapter 6, indicating a temporal factor affecting the survival and/or recovery of *P. cinnamomi*. The new inoculation technique employed has proved to be successful with *E. marginata* in earlier

work (Chapters 4 and 5) and with other species when used by other researchers (Dr. M. Dobrowolski *pers.comm*, T. Jackson, *pers. comm*. and Auckland (2002). However, minimal recoveries after the use of this technique were made from *Banksia hookerania* when temperatures were high (Auckland, 2002)

The temperature in the glasshouse was unexpectedly high on the day of inoculation, but surplus inoculum was successfully grown on agar when subcultured to confirm its viability. The maxima of ambient temperatures in the glasshouse at times, including inoculation and harvest days, exceeded the optima for *P. cinnamomi* survival and growth (Zentmyer, 1980; Hüberli *et al*., 1997), but it is likely that the inoculum, encased in the wet cotton wool support and bound with Parafilm to the stem, would have experienced a microclimate with temperatures lower than the recorded ambient temperatures. An additional observation with temperature probes in the cotton wool would support or reject this hypothesis, but if conditions were totally unfavourable for the development of the pathogen, even fewer recoveries would have been expected. The low percentage (36.7%) of recoveries from susceptible plants does indicate sub-optimal conditions for the development of disease caused by *P. cinnamomi.*

P. cinnamomi was not recovered from 6 of the 9 inoculated plants which died prior to harvest. The mortality of plants prior to harvest may have been due to droughted conditions. Though no control plants died in this trial, the death of 2 control seedlings in the previous trial (Chapter 6) indicates that it is possible that drought may have killed the inoculated plants. Containment and death (or dormancy) of the pathogen in the droughted, inoculated plants prior to their death may also explain the lack of recovery from these plants. Timing of inoculation, immediately prior to droughting, may also have been a factor contributing to the low recovery rate.

The extent of colonization of the susceptible (11J402) *E. marginata* stems was greater than that of resistant (1J30) *E. marginata* stems (Chapter 6) over a similar period of time (63 and 66 days, respectively) before harvest. Lesions were seen in 3 of the inoculated, susceptible, clonal plants but there were none seen in the resistant (Stukely and Crane, 1994; Cahill *et al.*, 1993) 1J30 clonal plants (Chapter 6).

The extent of colonization was not a determinant in the growth of plants. Invasive strategies can vary and an extensive colonization, tangentially, of the phloem and adjacent cortical region of a stem may have less effect on growth than a girdling of

the stem which will constrict vessels and totally impede water transport within the stem, killing the plant. The maximum increase in growth was seen in non-inoculated plants kept at container capacity. The reduced growth in the other inoculated treatments demonstrate the allocation of plant resources to defence rather than growth, coupled with less resources available in droughted conditions.

The variability of infection rates of *P. cinnamomi* inoculum and the variation in its recovery after leaching, even with pH and temperature adjusted and nutrients added to the leaching solution, need further research. Agar was not stained with exudates from the segments of most of the susceptible clones and leaching did not facilitate additional recovery of *P. cinnamomi* in this experiment, but was successful when using resistant plants (Chapter 6). It is possible that the lower levels of phenolic compounds in susceptible plants (Cahill *et al*., 1993) mean that leaching does not make an appreciable difference to the concentration of soluble phenolics in the plant tissue which inhibit the growth of *P. cinnamomi* onto the agar (Chapter 9).

7.4.2 *Other indications of stress contributing to development of disease*

Highest levels of stomatal conductance were recorded for non-inoculated plants kept at container capacity 10 and 23 days after inoculation, indicating they were less stressed than inoculated or droughted plants. Readings for non-inoculated plants kept at container capacity were significantly higher than inoculated plants in the same watering regime. Differences between inoculated and non-inoculated plants in the droughted treatments were not significant. Increase in the stomatal conductance of non-inoculated plants that were restored to CC after a period of drought, was greater (though not significantly so, $P>0.05$) than the increase in the inoculated plants in the same watering regime. These results could indicate that drought stress had more effect on stomatal conductance than did the development of the disease. This possibility was not supported by the results of the xylem pressure potential (XPP) at wilting point (WP). The mean XPP at WP was significantly lower for droughted inoculated plants than for droughted control plants which could indicate that the combination of drought and disease is greater than drought alone.

It has been reported that stomatal conductance in diseased *Eucalyptus* spp. was higher than comparable healthy plants initially, but decreased over time (Crombie and Milburn, 1988). However, in the current work, this was only seen for inoculated plants kept at CC, and not in the droughted plants. The interaction of drought and disease in the clonal plants susceptible to *P. cinnamomi* is open to interpretation. The trend for stomatal conductance of these susceptible *E. marginata* plants (11J402) to increase over time is the opposite trend recorded for resistant *E. marginata* plants (1J30) (Chapter 6).

7.4.3 *Conclusion*

Though the effect of watering regimes clearly indicates that *E. marginata* plants experiencing no water deficit are more extensively colonized by *P. cinnamomi* than droughted plants, the mortalities without pathogen recovery and the inconsistent results of the stress indicators (porometer readings and pressure chamber readings) challenge the conclusions drawn. Comparing the results of treatments of clonal plants resistant to *P. cinnamomi* (Chapter 6) or susceptible to *P. cinnamomi* (Chapter 7) with inconsistent variables of temperature, age and height of plants make some conclusions untenable. A further trial will be conducted with *E. marginata* plants inoculated with *P. cinnamomi* (Chapter 8) where seedlings and clonal plants, both resistant and susceptible clonal lines, will be subjected to the same watering regimes in the same conditions at the same time.

Chapter 8

Simulated summer rainfall and the development of disease caused by *Phytophthora cinnamomi* in droughted *Eucalyptus marginata* plants.

Part of the content of this chapter was presented as a poster at the Second International IUFRO Meeting, Albany, Western Australia, 2001.

Lucas, A., McComb J., Colquhoun, I.J. and Hardy G.E.St.J. (2001) Summer rainfall and the development of disease caused by *Phytophthora cinnamomi* in droughted *Eucalyptus marginata* plants.

A. Lucas conducted the research and prepared the poster. The other authors were the supervisors of the PhD project.

8.1 Introduction

Summer rainfall rarely occurs in the south-west of Western Australia but the consequence of these rare high summer rainfall periods is significant because the resulting warm, moist conditions favour an outbreak of *Phytophthora cinnamomi*. Many indigenous plant species, including the forest canopy species, *Eucalyptus marginata* (jarrah), are susceptible to *P. cinnamomi* when conditions are conducive (Podger, 1968; Zentmyer, 1980).

A field trial to compare the development of disease caused by *P. cinnamomi* in droughted and in irrigated *E. marginata* plants was interrupted by an unseasonal summer deluge which negated the effects of the drought treatment (Chapter 5). Separate glasshouse trials with clonal *E. marginata* plants resistant to *P. cinnamomi* (Chapter 6) and susceptible to *P. cinnamomi* (Chapter 7) compared the extent of colonization by the pathogen when plants were subjected to different watering regimes. Since the experimental parameters varied between these trials, the current study was designed to enable comparisons of *E. marginata* plants, resistant and susceptible to *P. cinnamomi*, grown under the same environmental conditions. The clonal lines used were the same as in the preceding chapters. In addition, *E. marginata* seedlings were concurrently subjected to the same watering regimes imposed on the clonal plants.

In previous glasshouse studies (Chapters 6 and 7), plants were inoculated prior to imposition of water deficit. This was modelled on seasonal increases of inoculum in the field during winter and spring rains (Shearer and Shea, 1987) which are normally followed by summer drought. However, infection in the field can occur after droughting, when the occurrence of a heavy summer rainfall event (Chapter 5) saturates soil, providing the conditions conducive to germination of surviving propagules of *P. cinnamomi* in the soil (Duniway, 1979; Zentmyer 1980). The current glasshouse study examines development of disease while simulating these field conditions, i.e. summer drought followed by a short period of flooding. Droughted plants were inoculated immediately after restoration of their substrates to container capacity (Fig. 8.1).

The aims of this experiment were to determine (i) whether plants which had experienced no water deficit were more susceptible to infection by *P. cinnamomi* than those which had been subjected to droughted conditions, and (ii) the extent of genotypic variation in the response of plants to infection by the pathogen.

The null hypotheses were (i) H_o: There will be no difference in the extent of colonization by *P. cinnamomi* in plants which have experienced droughted conditions immediately prior to inoculation and those plants which have not experienced drought stress and (ii) H_o: There will be no difference in the extent of colonization between plant genotypes in each watering regime.

8.2 Methods

8.2.1 *Experimental design*

In a completely randomized block design, 2 watering regimes (Fig. 8.1) were imposed on 3 *E. marginata* plant genotypes prior to inoculation with *P. cinnamomi* or sham inoculation, thus separating plants into 12 groups (Table 8.1). Half the plants of a clonal line (11J402) susceptible to *P. cinnamomi* (SS), and of a clonal line (1J30) resistant to *P. cinnamomi* (RR) (McComb *et al*., 1990; Cahill *et al*., 1992) and half the *E. marginata* seedlings (S) were kept at container capacity (CC) throughout the trial (watering regime 1). The other half were droughted to wilting point (WP), then had

10% of the moisture lost from CC to WP restored and they were maintained at this level for 3 weeks before restoration to CC for the remainder of the trial (watering regime 2) (Table 8.1). All plants were inoculated or sham inoculated immediately after the droughted plants were restored to CC, in conditions simulating summer rainfall after drought. They were then kept at CC by watering twice daily and harvested 14 days after inoculation (Fig. 8.1).

Table 8.1 Protocol for inoculation of *E. marginata* clonal plants susceptible to *P. cinnamomi* (SS), clonal plants resistant to *P. cinnamomi* (RR) and *E. marginata* seedlings (S) under 2 watering regimes (1) kept at container capacity and (2) droughted then restored to container capacity prior to inoculation (Fig.8.1).

Plant group	Plant type	Inoculated	Watering regime	Number of replicates
1	SS	+	1	8
2	SS	-	1	4
3	SS	+	2	8
4	SS	-	2	4
5	RR	+	1	8
6	RR	-	1	4
7	RR	+	2	8
8	RR	-	2	4
9	S	+	1	8
10	S	-	1	4
11	S	+	2	8
12	S	-	2	4

(+) = Inoculated with *P. cinnamomi*; (-) = sham-inoculated controls.

8.2.2 *Plant material and growing conditions*

All *E. marginata* clonal plants and seedlings were supplied by the Marrinup nursery of Alcoa World Alumina (Australia). Plants were grown in a peat/perlite mix (Appendix 1) in an evaporatively-cooled glasshouse until they had outgrown 150mm pots. When eighteen months old, they were transferred into a commercial native potting mix (Richgro Product # SSM 1590, Richgro Pty. Ltd., Canningvale, W.A. 6155) in 200mm free-draining, polyvinyl containers. All plants selected for the experiment had a well-defined periderm extending more than 10 cm from the base of a straight stem from which lower branches and coppice growth were removed. They were also tip-pruned and watered twice daily to container capacity for a month prior to commencement of the experiment. The trial was conducted in a glasshouse during the summer months of January and February. Maximum and minimum temperatures in the glasshouse were

recorded daily. Plant heights and stem diameters were recorded at commencement of the experiment (Height 1) and plant heights again at harvest or death (Height 2). Relative plant growth was calculated (Equation 8.1)

$$\text{Equation 8.1: Relative growth} = \frac{(\text{Height 2} - \text{Height 1}) / \text{days to death or harvest}}{\text{Height 1}}$$

8.2.3 *Inoculum preparation*

A virulent isolate of *P. cinnamomi* (MU 94-48) (Hüberli, 1995) was re-passaged through stem tissue of an *E. marginata* seedling (Appendix 9). After harvesting, stem segments were plated onto sterile NARPH selective agar (Hüberli, 2000) in 90mm Petri dishes. Outgrowth of *P. cinnamomi* from these infected segments was sub-cultured and grown on plates of sterile V8 agar (Appendix 2a) for 7 days. Incisions made in the colonized agar in a grid of 1cm x 1cm cuts, resulted in mycelial inoculum plugs of uniform dimensions. Plugs of sterile non-colonized V8 agar of the same size were prepared as sham inocula for the control plants.

8.2.4 *Inoculation*

The non-wounding inoculation technique, developed in Chapter 4 and described for the glasshouse trial in Chapter 6.2.5, was applied.

8.2.5 *Watering regimes*

Two watering regimes were imposed prior to inoculation (Fig. 8.1). The substrates of half the clonal plants and half the seedlings were kept at container capacity (CC) by hand-watering twice daily throughout the trial. The substrates of the other half were brought to CC, then droughted to wilting point (WP) by withholding water. Replenishment of 10% of the moisture lost from CC to WP brought them to the droughted level (determined in Chapter 2) at which they were maintained for 3 weeks. They were then restored to CC. The first watering regime kept plants in a non-stressed condition, without water deficit. The second watering regime simulated summer rainfall after a period of drought stress. Plants were inoculated or sham-inoculated immediately after restoration to CC.

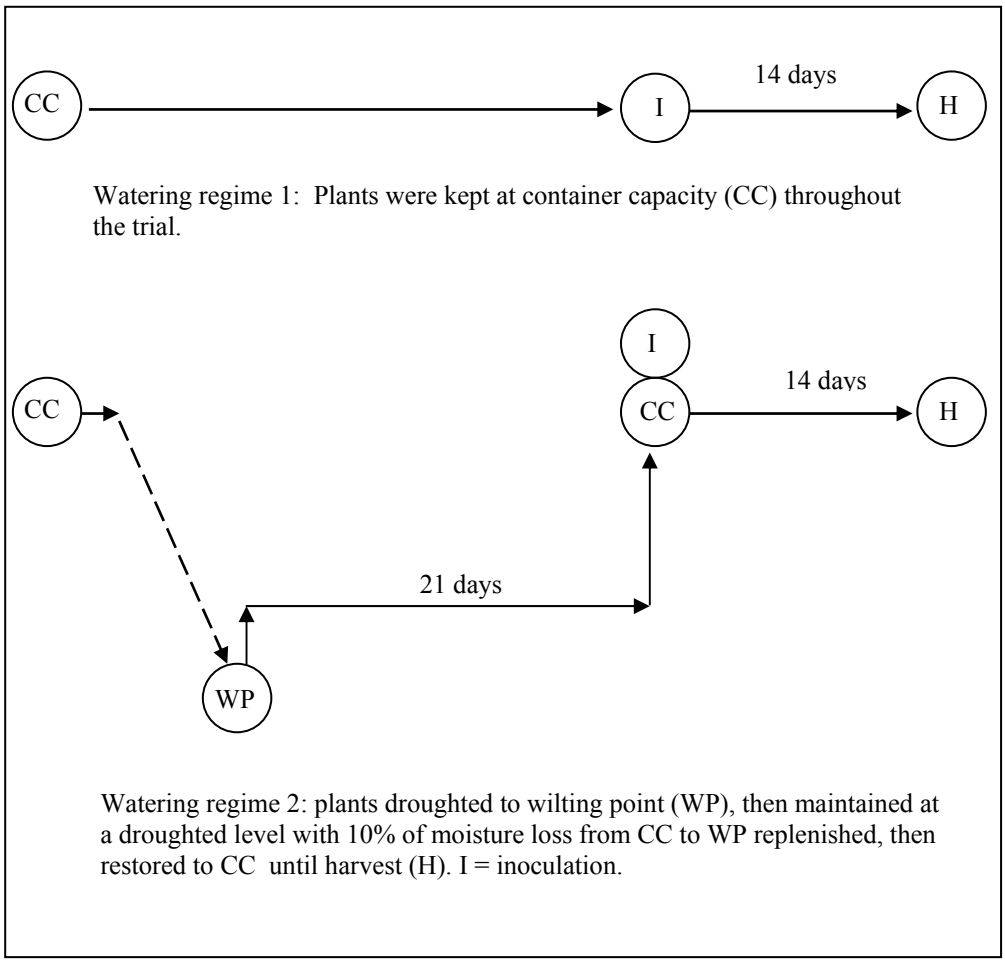


Figure 8.1 Schematic diagram showing the two watering regimes imposed on inoculated *E. marginata* plants. Control plants were subjected to the same watering regimes and sham-inoculated.
CC = Container capacity; WP = Wilting point; I = Inoculation; H = Harvest.

8.2.6 *Evaluation of plant stress*

Stress levels of plants were monitored using 4 different methods.

1. Daily weighings recorded moisture loss of container substrate and the number of days to WP of each droughted replicate was noted.

2. Visual inspections of leaves and stems gave an indication of a plant's physiological response and plants were considered to be at WP when the tips of new shoots were

no longer turgid. Developing lesions, chlorotic conditions and pre-harvest mortality due to drought or disease were noted.

3. Xylem pressure potential was recorded at wilting point using a pressure chamber similar to that described by Scholander *et al*. (1965).

4. A series of porometer readings (LI-COR LI-1600 Steady State Porometer) on the youngest fully expanded leaf (YFEL) of the plant, marked with thread to ensure the same leaf was read at each stage, calculated the stomatal conductance at 6 crucial time intervals; (1) at the start, (2) when all plants had been prepared and maintained at CC for 7 days, (3) while droughted, (4) on the day of inoculation after being restored to full CC, (5) 7 days after inoculation and (6) immediately prior to harvest.

8.2.7 *Harvest*

Plants were harvested 14 days after inoculation. Visible surface lesions were measured from the ROI. Stems were cut in 1 cm segments from 20 cm above the lesion front and down to the front. If no lesion was visible, segments were cut from 20 cm above, and down to, the ROI. These numbered segments were plated onto NARPH selective agar in 90 mm Petri dishes, sealed with Parafilm and placed in the dark at 24±1^{o}C. Plates were inspected daily for 14 days for the presence/absence of the pathogen and if recorded as present, the infected segment was removed with the surrounding colonized agar before the plate was returned to the incubator. Plants which died prior to inoculation, or after inoculation and prior to harvest, were also processed and plated onto selective agar, as above, to determine the presence or absence of *P. cinnamomi.*

8.2.8 *Recovery of* P. cinnamomi

The stem segments from which *P. cinnamomi* grew onto agar were noted. Segments of stems from which there was no initial recovery of *P. cinnamomi* were removed from the agar and immersed in sterile distilled water in McCartney bottles. The water was changed each day, until no discolouration occurred. Stem segments were

dried with sterile absorbent paper and replated onto NARPH selective agar. Records of any additional recoveries were added to the original data.

8.2.9 *Statistical analysis*

Data were analysed using Statistica® for Windows Version 5 (StatSoft Inc., OK, USA) and Excel for Windows (Microsoft Corporation, USA), Microsoft Excel Analysis ToolPak (GreyMatter International, Inc., Cambridge, MA 02141). Prior to parametric analyses, data were screened for basic assumptions of parametric tests including normal distribution, equality of variances and non-correlation of means and variances. In cases where data did not conform to assumptions, log transformations were used to correct the deviations. The use of such transformations is noted in the relevant results. Where data were log-transformed, means are presented graphically for non-transformed data. After comparison of data between or within plant groups (Table 8.1), where an ANOVA resulted in a P-value of < 0.05, data were considered significantly different.

8.3 Results

8.3.1 *Plant growth and temperatures in the glasshouse*

There was no significant (*df* 11, 60; $P > 0.05$) difference in plant heights or stem diameters between the 12 groups (2 watering regimes x 3 genotypes x 2 inoculation treatments) of plants at the start of trial. Relative plant growth during the experiment (Equation 8.1) was greater in both inoculated and control plants in watering regime 1 (plants kept at container capacity) than in watering regime 2 (droughted plants) in the 3 genotypes (Figure 8.2). Within each genotype and between genotypes, there was no significant ($P > 0.05$) difference in the relative growth between inoculated and control plants nor between plants kept at CC and droughted plants. In both watering regimes, least increase in growth was observed in RR clonal plants (Fig. 8.2).

The experiment was conducted in the summer months of January and February when maximum daily temperatures in the glasshouse ranged from 28.4°C to 36.2°C (mean 32.5°C) and minima from 18.0°C to 25.7°C (mean 23.6°C).

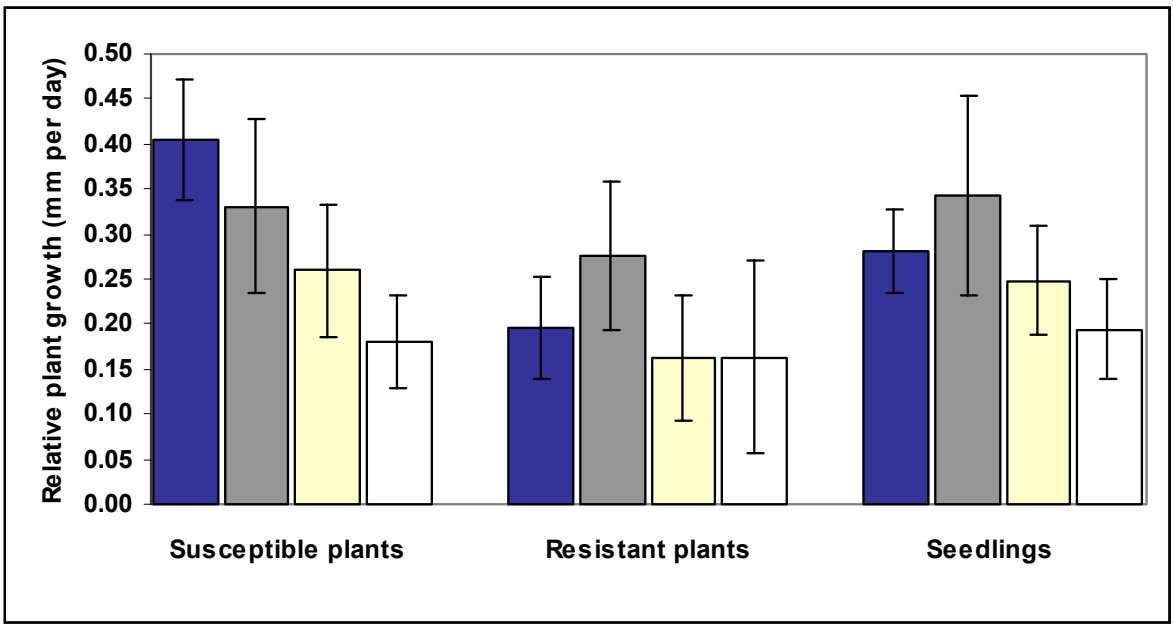


Figure 8.2 Relative growth rate of *E. marginata* plants of 3 different genotypes in 2 watering regimes, relative to height at the start of the experiment. Bars represent the standard error of the mean.

Inoculated plants in watering regime 1, kept at container capacity (CC).

Non-inoculated control plants in watering regime 1.

Inoculated plants in watering regime 2, droughted then restored to CC.

Non-inoculated control plants in watering regime 2.

8.3.2 *Wilting point*

Droughted plants took from 11 to 22 days to reach WP and SS clones took longer than the other plant types (Table 8.2). XPP at WP ranged from –2.00 to –4.8 MPa and while seedlings recorded greatest water stress with most negative XPP (Table 8.2), there was no significant (P>0.05) difference between the three plant genotypes, nor between resistant and susceptible clonal plants. Poor correlation was found between the

number of days to WP and the XPP at WP and within groups (Table 8.2) and also between all replicates (r = 0.296).

Table 8.2 Mean number of days for each droughted plant genotype to reach wilting point (WP) and the correlation between that and xylem pressure potential (XPP) at WP.

	Mean number of days to WP	XPP at WP (MPa)	Correlation r =
SS (n = 12)	18.75 ± 0.79	-3.17 ± 0.23	0.405
RR (n = 12)	16.33 ± 0.69	-2.86 ± 0.12	0.502
S (n = 12)	15.92 ± 0.77	-3.29 ± 0.17	0.247

SS = clonal plants of *E. marginata* (11J402), susceptible to *P. cinnamomi*;
RR = clonal plants of *E. marginata* (1J30), resistant to *P. cinnamomi*.
S = Seedlings of *E. marginata*.

8.3.3 *Stomatal conductance*

Stomatal conductance could not be read on the leaves of dead and some chlorotic plants at harvest. In inoculated and in non-inoculated SS plants in watering regime 2 (droughted), only one reading was possible at harvest (reading 6). The other 10 plant groups had 3 or more surviving plants, which were read. In the inoculated plant groups, only the readings of plants from which *P. cinnamomi* was recovered were included in the 3 replicates selected for statistical analysis of stomatal conductance. By comparing readings from the same 3 plants, a valid comparison could be made over time. A repeated measures analysis showed no significant (*df* 5, 100; P > 0.05) effect of genotype, inoculation status or watering regime.

Mean stomatal conductance readings, for inoculated replicates of the 3 genotypes (SS, RR and S) in watering regime 2 (droughted then restored to container capacity (CC)), over time showed that higher values were recorded at CC, prior to droughting (first and second readings) (Fig. 8.3). At the time of inoculation when the plants were again at CC, there was some increase. There was a drop in stomatal conductance 7 days after inoculation but when the plants were harvested, the stomatal conductance was as high as at the beginning of the experiment. These readings were then compared to the readings of non-inoculated control plants in watering regime 1

(kept at CC throughout the trial). It would be expected that the non-inoculated and non-droughted control plants, while kept at CC throughout the trial would maintain a higher level of stomatal conductance without the fluctuations recorded for the inoculated plants, droughted then restored to CC. However, a similar pattern was recorded (Fig. 8.4). Mean stomatal conductance for inoculated and control plants, of all genotypes in both watering regimes, was lower at reading 3 (when some plants were droughted) and at reading 5 (7 days after inoculation). There was a limited range of maximum temperatures in the glasshouse when porometer readings were taken (Fig. 8.5).

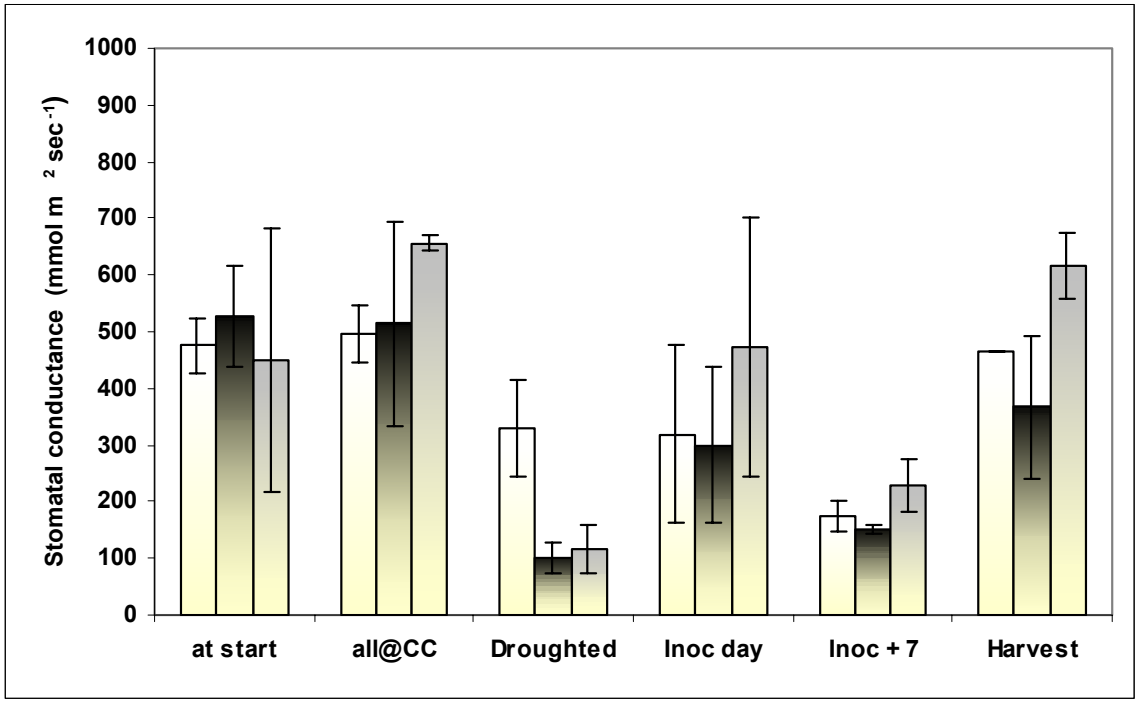
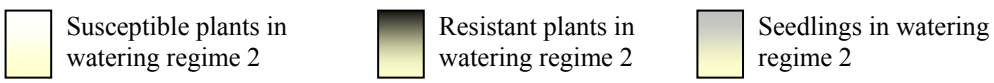


Figure 8.3 Mean stomatal conductance of inoculated *E. marginata* plants, which survived until harvest, in watering regime 2, plants droughted, then restored to container capacity immediately prior to inoculation (n = 3, except droughted, susceptible plants at harvest where n = 1). Bars represent the standard error of the mean.



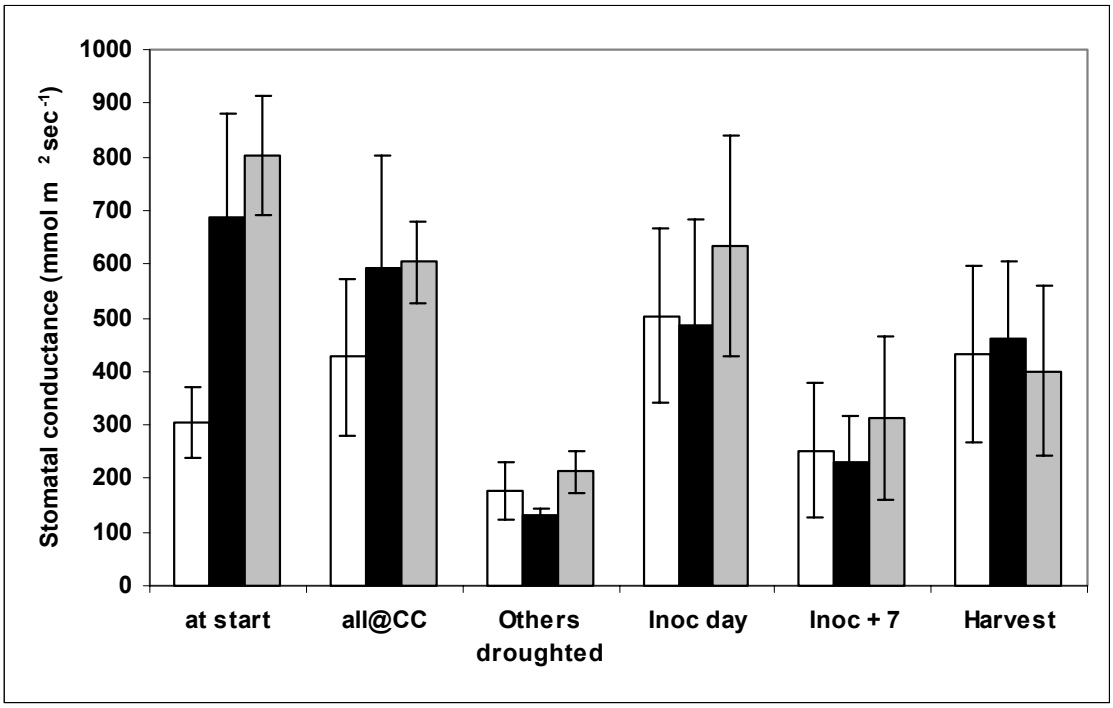


Figure 8.4 Mean stomatal conductance of non-inoculated *E. marginata* control plants in watering regime 1, plants kept at container capacity (CC) throughout the trial (n = 3). Readings were taken at the same time as the plants shown in Fig. 8.3 which were inoculated and droughted. Others droughted = plants in watering regime 2 were droughted at this time. Bars represent the standard error of the mean.

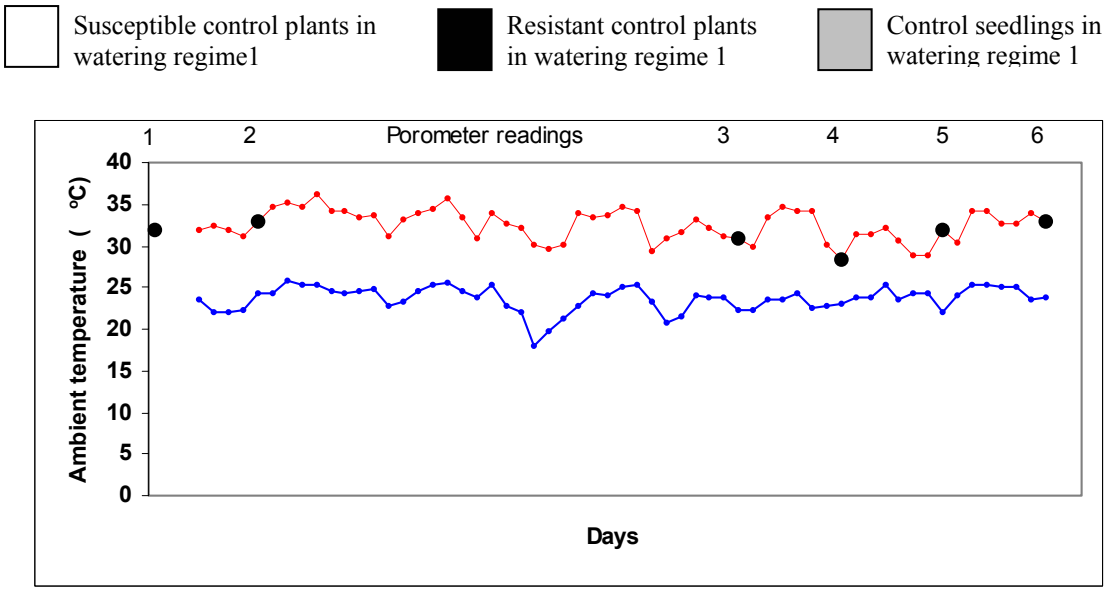


Figure 8.5 Maximum (red) and minimum (blue) temperatures in the glasshouse in January and February, with black points indicating maximum temperature on days of porometer readings. Porometer readings: 1 = prior to start of experiment, 2 = when all plants were at container capacity, 3 = when half the replicates were droughted, 4 = when plants were inoculated, 5 = 7 days after inoculation, 6 = at harvest.

8.3.4 *Mortality prior to harvest*

There were 8 deaths prior to inoculation. All were droughted (watering regime 2) and no *P. cinnamomi* was recovered from these plants. Four more droughted plants died after inoculation but before harvest. These plants had been noted as dying when inoculated. *P. cinnamomi* was recovered from 2 of these (Table 8.3). No *P. cinnamomi* was recovered from any non-inoculated control plants in any group.

Table 8.3 Number of deaths of *E. marginata* plants subjected to two watering regimes, prior to inoculation also after inoculation and prior to harvest.

	Watering regime 1		Watering regime 2		
Plant type	dead prior to inoculation	dead after inoculation and prior to harvest	dead prior to inoculation	dead after inoculation and prior to harvest	
				inoculated	controls
SS	0	0	5	**1**	0
RR	0	0	1	2	0
S	0	0	2	**1**	0

Numbers in bold indicate recoveries of *P. cinnamomi* from dead plants.
Watering regime 1 = plants kept at container capacity (CC) throughout trial.
Watering regime 2 = plants droughted, then restored to CC as in Fig. 8.1
SS = *E. marginata* clonal plants (11J402) susceptible to *P. cinnamomi*; RR = *E. marginata* clonal plants (1J30) resistant to *P. cinnamomi*; S = *E. marginata* seedlings.

8.3.5 *Recovery of* P. cinnamomi *prior to and after leaching*

Recovery of *P. cinnamomi* was made from all inoculated plant genotypes, with more recoveries from the stems of plants in watering regime 1 (plants kept at CC) both prior to and after leaching (Table 8.4). No *P. cinnamomi* was recovered from any sham-inoculated control plants.

Table 8.4 Recovery of *P. cinnamomi* from *E. marginata* stems in 3 plant genotypes, subjected to 2 different watering regimes. Results include recoveries made from plants which died prior to harvest.

	Watering regime 1			Watering regime 2			
	SS	RR	S	SS	RR	S	Totals
Stems inoculated	8	8	8	8	8	8	48
Recoveries prior to leaching	5	5	5	4	4	5	28
Additional recoveries after leaching	2	1	0	0	0	0	3
No recovery Inoc. stems dead	0	0	0	3	3	2	8
No recovery Inoc. stems alive	1	2	3	1	1	1	9
Total recoveries	7	6	5	4	4	5	31

Watering regime 1 = plants kept at container capacity (CC) throughout the trial.
Watering regime 2 = plants restored to CC after droughted conditions.
SS = *E. marginata* plants of a clonal line (11J 402) susceptible to *P. cinnamomi*
RR = *E. marginata* plants of a clonal line (1J30) resistant to *P. cinnamomi*
S = *E. marginata* seedlings. Inoc. = inoculated.

8.3.6 *Lesions and colonization*

No surface lesions were observed on the main stem of any plants above the ROI. Lesions, below the level of the ROI, were observed on coppice stems of one seedling in watering regime 2 (droughted) and one clonal plant (1J30) resistant to *P. cinnamomi*, in watering regime 1 (kept at CC).

The extent of colonization at and above the ROI in all groups was compared. The 8 droughted plants in watering regime 2 which were dead prior to inoculation were excluded from the statistical analysis of total colonization. One outlier (>2.5 of the mean) was excluded from the data for droughted, inoculated seedlings in watering regime 2. All data of total colonization (x+1) were logarithmically transformed to meet the assumptions required by an ANOVA.

A 2-way ANOVA of total colonization of inoculated plants showed no significant ($P > 0.05$) effect of genotype or watering regime. However, there were two clear trends. Firstly, within each genotype, plants in watering regime 1 (kept at CC)

were more extensively colonized by *P. cinnamomi* than plants in watering regime 2, which had been droughted prior to inoculation, and secondly, susceptible plants in each watering regime were more extensively colonized than resistant plants or seedlings in the corresponding watering regime (Fig. 8.6). The standard error bars indicate a high degree of variability of colonization by *P. cinnamomi* within each genotype (Fig. 8.6).

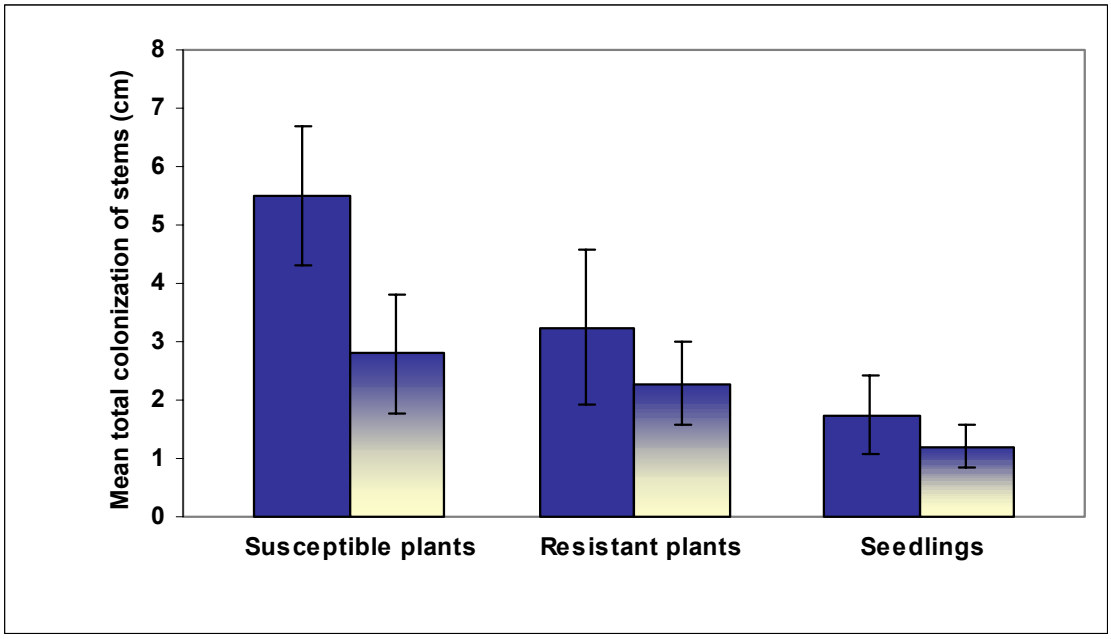


Figure 8.6 Mean total colonization of *E. marginata* stems by *P. cinnamomi.* Bars represent the standard error of the mean.

Watering regime 1 Inoculated seedlings kept at container capacity (CC) throughout the trial.

Watering regime 2 Inoculated seedlings droughted to wilting point (WP) then maintained until harvest at a droughted level, with 10% of substrate moisture lost from CC to WP restored and restored to CC after 14 days of droughted conditions.

8.4 Discussion

8.4.1 *A comparison of the response to* P. cinnamomi *of plants subjected to two different watering regimes.*

Drought stress reduced colonization by *P. cinnamomi* in both resistant and susceptible *E. marginata* clonal plants and in seedlings. These results support the findings of Chapters 3, 6 and 7. More recoveries of *P. cinnamomi* were made from resistant and susceptible clonal plants kept at container capacity (CC) throughout the trial (watering regime 1) than from previously droughted plants restored to CC (watering regime 2) with an equal number of recoveries from seedlings.

Despite this, there were no deaths of plants in watering regime 1 (plants kept at CC), but 12 deaths in watering regime 2 (plants droughted then restored to CC). Of the 12 deaths, 8 died prior to inoculation and these deaths can be attributed to drought since no *P. cinnamomi* was recovered from these non-inoculated plants. The level of water deficit imposed depended on the weight at wilting point, which was determined by the subjective observation of loss of turgidity in leaf tips. The replacement of 10% of moisture loss from CC to WP was sufficient for young and healthy seedlings (Chapter 2) but may not have been for the older plants with higher rates of transpiration. The rate of transpiration of *E. marginata* recorded by other researchers has also been high both in the field (Doley, 1967; Colquhoun *et al*., 1984) and in the glasshouse (Stoneman *et al*., 1994). All 4 inoculated plants which died prior to harvest had been noted as dying at inoculation, so, even though *P. cinnamomi* was recovered from 2 of them, their deaths may also have been the result of a too severe water deficit, rather than disease.

In Chapters 6 and 7, drought and disease stress were concurrent, making it difficult to determine which of the two stressors caused death. In contrast, in the current study, drought and disease were not concurrent, as plants were restored to full container capacity prior to inoculation. Plants in watering regime 2 can be considered to be 'previously droughted' rather than droughted during pathogenesis. It has been suggested that drought stress may predispose plants to pathogenic invasion (Schoeneweiss, 1975; Boyer, 1995). Most deaths were prior to inoculation and therefore likely to be due to

droughted conditions, since no *P. cinnamomi* was recovered from them. Deaths which occurred after inoculation and prior to harvest can reasonably be ascribed to the disease.

All plants (those previously droughted and those kept at CC throughout the trial), were kept at CC after inoculation. The restoration of moisture to plant tissue may have been sufficient to allow *P. cinnamomi* to colonize the host tissue rapidly and cause disease. This was observed in more than half the droughted plants. This resembled the field conditions (Chapter 5) when the sudden summer rainfall and warm weather initiated the spread of inoculum, from the soil or from nearby host plants, causing the death of seedlings

However, in the current glasshouse trial, *P. cinnamomi* was not introduced until after the droughted period when all plants were at full container capacity. For some replicates, this simulated summer rainfall after drought. Consequently, this experiment more closely resembled a summer rainfall event where plants become infected as a result of this event. In contrast, the earlier studies (Chapters 6 and 7) represent a summer rainfall event where plants have been previously infected during spring or autumn, and the summer rainfall facilitates the breakout of the pathogen from previously contained lesions in stems with low bark moisture. The sudden introduction of *P. cinnamomi*, with a simultaneous increase in substrate moisture, is a possible scenario in the ponded riplines of rehabilitated minesites of Western Australia when stems, collars or lower branches of plants may be submerged. Seasonal factors influence the levels of inoculum (Shearer and Shea, 1987), and depending on the topography of the site, inoculum can be transported through the soil (Kinal *et al*., 1993) or aboveground in water flowing along riplines (O'Gara 1998). The subsequent rapid infection of plants in a rehabilitated mine site has been demonstrated (Chapter 5).

Why were the previously droughted plants less susceptible to colonization by the pathogen? Tippett and Hill (1983) found a correlation with higher levels of bark moisture and more extensive development of *P. cinnamomi* lesions in *E. marginata* and Bunny *et al*. (1995) reported greater lesion development in higher rainfall sites. Higher bark moisture of the host means that the mycelial growth of *P. cinnamomi* is facilitated. While the importance of timing of defence responses can be considered in the following section, are newly infected plants with high moisture content less able to produce phenolic compounds? Does lower moisture content of plant tissue in droughted

replicates lead to a subtle but effective increase in aeration *in planta* and the oxygen needed for the synthesis of phenolic compounds used in the defence strategies of the plant? Though outside the experimental design of the current study, this concept may also apply to previously droughted plants restored to container (or field) capacity.

The results of the current glasshouse study imply that, in seasons when plants have experienced the droughted summer conditions which usually occur in the mediterranean climate of the south-west of Western Australia, potential hosts will be less susceptible to colonization by the pathogen than plants which are infected in autumn through to the end of spring when rainfall has provided soil moisture for uptake by plants. However, the timing of planned sowing and planting for revegetation projects on soil infested with *P. cinnamomi* is limited to times that coincide with the first rains to assist the establishment and survival of young plants.

8.4.2 *A comparison of the response of different genotypes of* E. marginata *to* P. cinnamomi *and watering regimes.*

Greatest extent of colonization was found in clonal plants susceptible (SS) to *P. cinnamomi*. In the previous studies, the SS plants (Chapter 7) were also more extensively colonized than the resistant (RR) clonal plants (Chapter 6), though the number of days to harvest after inoculation was more than 60 in the two previous studies, compared to 14 days in the current study. However, in the current study, though plants were harvested 14 days after restoration to CC (*cf* 21 days in Chapters 6 and 7), plants were inoculated after the droughted period, reducing the number of days from inoculation to harvest. Resistant plants of *E. marginata* have higher levels of phenolic compounds (Cahill *et al*., 1993) and may be able to synthesize lignin and suberin faster than the SS plants after detection of the pathogen. The deposition of these polymers into cell walls forms a structural barrier (Vance *et al*., 1980; Bostock and Stermer, 1989) and if the rate of deposition is fast then there is less opportuninty, spatially and temporally, for the pathogen to colonize the host tisssue (Bowles, 1990). This can apply to the RR *E. marginata* clones, which may respond faster than the SS clones and some seedlings in recognition of and defence against the pathogen.

Most deaths were in SS plants, droughted prior to inoculation (watering regime 2), with an additional death prior to harvest. There were no deaths of plants of any

genotype kept at container capacity (watering regime 1), though colonization was greater. Recoveries of *P. cinnamomi* were similar for all genotypes in the current study (62-68%) when the results for both watering regimes were combined. Recoveries of *P. cinnamomi* in previous studies of RR clonal plants (Chapter 6) and SS plants (Chapter 7) were also similar with 35% and 36%, respectively. Harvesting plants 14 days after inoculation (current study) instead of more than 60 days after inoculation (Chapters 6 and 7) may have contributed to the higher proportion of recovery made from both RR and SS plants. Other researchers have found that recovery of *P. cinnamomi* from the host tissue diminishes with time when plating harvested plants onto agar (Davison *et al*., 1994; O'Gara 1998; McDougall *et al.,* 2002).

The mean number of days for all droughted SS clonal plants to reach wilting point was slightly higher than the mean for RR plants or for seedlings in the current study. This could indicate a higher level of plant moisture in the SS plants. This suggestion is not supported by the results of previous studies where the mean for SS plants (Chapter 7) was one day less than that for RR plants (Chapter 6), but a contributing factor in this comparison may be the glasshouse temperature (Hüberli, 2002; R. Pilbeam *pers. comm*.) which was higher during the experiment with SS plants. In the current study, all environmental factors were the same for all plants and differences observed within treatments, may be attributed to genotypic response.

8.4.3 *A comparison of the response to different timing of inoculation*

Timing of the inoculation in relation to imposition of watering regimes is an important factor to consider. In previous studies with RR plants (Chapter 6) and SS plants (Chapter 7), plants were droughted by withholding water immediately after inoculation but in the current study, plants were not inoculated until after the droughted period and immediately after plants had been restored to container capacity. This simulated the situation in the field at the time of a summer rainfall event. In all experiments some plants had been kept at container capacity throughout the trial.

More colonization was seen in SS plants and a higher proportion of recoveries of *P. cinnamomi* were made in the current study compared to Chapter 7 where plants were inoculated prior to droughting. Similarly, more colonization was seen in RR plants and

a higher proportion of recoveries of *P. cinnamomi* were made in the current study than in Chapter 6 where plants were inoculated prior to droughting. These results suggest that the pathogenic ability of *P. cinnamomi* declines as the water content of its host declines during drought and increases when substrates are suddenly restored to container (or field) capacity. This supports the observations made in the field study (Chapter 5) and other research on development of *P. cinnamomi* lesions when host plants experienced water deficit (Smith and Marks, 1986; Tippett *et al*., 1987), or droughting and rewatering (Marcais *et al*., 1993; Robin *et al*., 2001).

Less colonization was seen in the seedlings in the current study than in the RR or in the SS clonal plants of Chapters 6 and 7. This could indicate that they were a either a very resistant batch of seedlings or that the age difference of the seedlings in the current study (24 months) compared to the RR and SS clonal plants (18 and 20 months, respectively) in Chapters 6 and 7 gave them a physiological advantage with more developed rhytidome (O'Gara, 1998). However, there was also less colonization in these seedlings than in the clonal plants in the current study, which were of the same age and which were not significantly different in height to the seedlings. Results for one inoculated and droughted plant, which was statistically an outlier, was removed from the data, reducing the mean for that group, but the extent of colonization was low in the inoculated seedlings which had been kept at container capacity, inferring a high level of resistance in the seedlings. Droughted inoculated seedlings recorded higher levels of stomatal conductance than SS or RR at each reading from inoculation to harvest.

8.4.4 Conclusion

This study showed some clear differences in disease development between *E. marginata* genotypes in response to different watering regimes. The inclusion of both RR and SS clonal lines of *E. marginata* plants, and seedlings in the same glasshouse trial allowed more valid comparisons to be made within this study than comparisons between previous studies (Chapters 6 and 7). A power test showed that statistically significant differences in analyses of data would be more likely to be shown with a higher number of replicates (at least 50) in each treatment. Variables of light,

temperature, substrate, water deficit, inoculation technique, age and condition of inoculum isolate and days to harvest, were all standardized between plant groups in the controlled conditions of the glasshouse. However, some comparisons can be made with previous studies and there is good evidence for greater colonization of plant tissue by *P. cinnamomi* in plants which do not experience water deficit. While results obtained in the glasshouse may not be able to be extrapolated to field conditions, the results reiterated in the glasshouse trials are supported by observations in the field. After heavy summer rainfall in January 2000, the spread of disease from inoculum in the soil was rapid, causing the death of *E. marginata* seedlings and clonal plants resistant to *P. cinnamomi.* Though the establishment of root systems and survival of germinating seeds restricts the timing of planting and sowing in revegetation projects on mine sites (Ward *et al*., 1996), there are implications for management of commercial crops, e.g. Proteaceae, susceptible to *P. cinnamomi*, which are irrigated during the summer months.

Chapter 9

Investigation of factors which inhibit or facilitate the *in vitro* growth of *Phytophthora cinnamomi* and its recovery from infected stems of *Eucalyptus marginata.*

9.1 Introduction

The accurate assessment of the presence or absence of *Phytophthora cinnamomi* in host plants is important for forest and nursery management, mine site rehabilitation and for decisions regarding quarantine. Inaccurate diagnosis can result in the introduction of a symptomless, but *P. cinnamomi*-infected host to otherwise disease-free areas. It can also result in the incorrect assessment of an area as disease-free. Previous studies (Crombie and Tippett, 1990; O'Gara, 1998; Hüberli *et al*., 2000) and the present work (Chapter 5) have observed that the pathogen was not recovered from some inoculated stems which had clearly visible surface lesions, typical of *P. cinnamomi*. It is important to understand the mechanisms which prevent the recovery of *P. cinnamomi* and to devise reliable methods that facilitate its recovery.

It is possible that soluble phenolics and other compounds in the plant tissue inhibit the isolation of the pathogen. Repeatedly washing and rinsing infected stem segments which initially did not reveal the presence of the pathogen after direct plating onto selective agar, has led to further recovery of *P. cinnamomi*. Increased recovery has been made from 8% to 11% of stems, initially recorded as disease-free (Hüberli *et al*., 2000). This lack of recovery using traditional isolation methods from obviously infected plants suggests that recovery techniques are not sufficiently reliable to assess the full extent of the spread of the pathogen in field surveys. Failure to isolate the pathogen could be because it has been inhibited by phytoalexins or phenolic compounds or has died after its spread *in planta* has been contained by the host. The pathogen may have entered a dormant state (such as chlamydospores) and the current recovery techniques are unable to break the dormancy. The improvement in recovery by leaching away inhibitory compounds is a significant first step, but to ensure accurate recoveries of the pathogen from plant material it is important to understand why *P. cinnamomi* is not always recovered from infected tissue.

Molecular diagnostic methods are proving useful in the detection of pathogens, but several standard recovery procedures are still employed by many researchers, including surface sterilization of root or stem segments, prior to plating onto selective agar, followed by incubation of the samples at optimal temperatures and monitoring the growth on the agar. A range of media, selective for *Phytophthora*, has been developed to detect its presence in plant tissue (Tsao and Ocana, 1969; Masago *et al*., 1977; Kannewischer and Mitchell, 1981; Tsao and Guy, 1977; Jeffers and Martin, 1986; Shearer and Dillon, 1995; Hüberli *et al*., 2000). Identification of the pathogen can then be confirmed microscopically using taxonomic keys. Clearly these isolation techniques need to be modified to give more accurate diagnosis of the presence of the pathogen.

While constitutive phenolics in plants can deter herbivory, it is the *de novo* synthesis of phenolic compounds that can play a role in the plant's defence against invasion by a pathogen (Bennett and Wallsgrove, 1994). The sensitivity of *Phytophthora* spp. to different phenols varies (Caseres *et al*., 1986; Cahill and McComb, 1992). Oxidized phenolics can be fungitoxic (Retig, 1974) and when the levels of peroxidase and polyphenoloxidase rise *in planta* in response to pathogen attack (Retig, 1974; Okey *et al*., 1997; Burgess *et al*., 1999a), some phenolic compounds will be oxidized and become more toxic to pathogens (Kosuge, 1969: Dimond 1970). Higher levels of phenolic compounds have been found in *E. marginata* plants resistant to *P. cinnamomi* (Cahill *et al*., 1993). In routine recovery of *P. cinnamomi* from infected stems of *E. marginata*, compounds frequently exude from inoculated plant tissues onto the selective agar medium on which the stem segments are plated, with the characteristic staining of tannins and other phenols. Not only is detection of any mycelial growth of *P. cinnamomi* impaired by these exudates, but also the growth is often less vigorous than that from tissues where no exudates were released. It was hypothesized that, if soluble phenolic compounds[1] oxidise when exposed to air and suppress the growth of the pathogen from the plant tissues onto the agar medium, then the introduction of an antioxidant into the selective agar medium used for recovery of *P. cinnamomi* could counter this inhibitory effect.

[1] The presence of phenolic compounds (known to occur in *E. marginata*) in exudates from the infected stems was suggested by the characteristic dark brown staining. Exudates will sometimes be referred to as phenolic compounds in this chapter.

Several separate, but linked experiments which investigated the role played by temperature, pH, soil microbes, phenolic compounds and antioxidants in the recovery of *P. cinnamomi* from *Eucalyptus marginata* (jarrah) were examined in this chapter. Factors that inhibit the growth of the pathogen, *in planta* and *in vitro*, as well as methods that counter inhibitory effects, were examined.

The aims of the experiments in this chapter were to evaluate conventional recovery techniques and to modify them, providing conditions more suitable for the *in vitro* growth of *P. cinnamomi*. This would facilitate not only the initial detection of *P. cinnamomi* in plant tissue but also subsequent detection in plant tissue previously observed to be pathogen-free, thereby reducing the number of false negatives assigned to samples of diseased plants. While Methods and Results of each trial are presented separately, the Discussion at the end of this chapter considers all experiments.

9a Recovery of *Phytophthora cinnamomi* from a clonal line of resistant *Eucalyptus marginata* plants.

9a.1 Introduction

In a previous field experiment (Chapter 5) with a clonal line (77C40) of *E. marginata* plants resistant (M. Stukely, *pers. comm*.) to *P. cinnamomi*, segments from inoculated stems with visible surface lesions typical of *P. cinnamomi* infection, had been cut and plated onto selective agar. The segments had been cut from above the lesion front and down to it (to avoid contamination) and also from the 1 cm section of stem that was the region of inoculation (ROI) (Fig. 9a 1-1). Remaining sections of the lesioned stem below the lesion front (Fig 9a.1-1) were kept at 4^{o}±1C for 7 days, while the cut segments were monitored for presence of the pathogen (Chapter 5). *P. cinnamomi* was not recovered from some of these stems with surface lesions (Chapter 5) (Fig. 9a.1-2). It was noted that exudates from these stem segments stained the agar onto which they had been plated a dark brown. Six of the inoculated stems from which no *P. cinnamomi* was recovered were selected for further experiments (Table 9a.1).

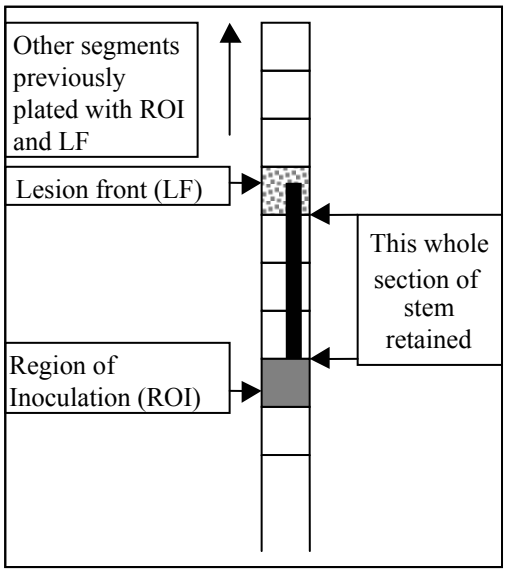


Figure 9a.1-1 Diagram of the lesioned stem. The region of inoculation (ROI), lesion front (LF) and segments above the lesion front were plated onto selective agar immediately after harvest. The thick black line represents the lesion.

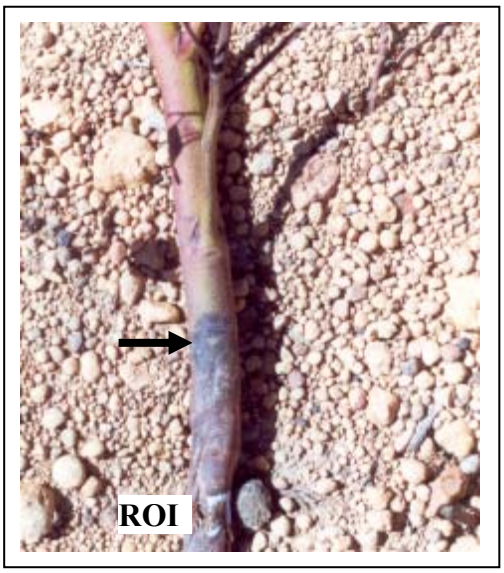


Figure 9a.1-2 Harvested stem of an *E. marginata* plant of a clonal line (77C40) resistant to *P. cinnamomi*. Lesion (arrowed) is clearly visible above the periderm. Diameter of stem at region of inoculation (ROI) was 3.07 cm.

The aim of this experiment was to determine if, with further treatment, recovery of the pathogen could be made from lesioned stems which had been inoculated with *P. cinnamomi* and from which no recovery had been made with the initial direct plating onto selective agar. (These were not the same segments but the same stems.)

9a.2 Methods

9a.2.1 *Experimental design*

Six *E. marginata* stems, inoculated with *P. cinnamomi*, but from which the pathogen was not recovered after direct plating onto selective agar despite the presence of lesions typical of *P. cinnamomi* colonization, were used. Segments from 2 of the stems were treated individually, while segments from the remaining 4 stems were bulked and treated as a mix. Stem 1, Stem 2 and Stem Mix were all subjected to 4 treatments, after leaching for 3 days in either sterile distilled water (SDW) or soil filtrate (SF) (Table 9a.1). After treatment, segments from the individual stems and the stem mix were plated onto selective agar in 5 replicate Petri dishes. Stem segments from non-

inoculated *E. marginata* plants were used as controls and after treatment, plated onto 3 replicate Petri dishes.

Table 9a.1 Protocol for recovery of *P. cinnamomi* from segments of inoculated and lesioned stems of *E marginata* plants from which the pathogen was not recovered when directly plated onto selective agar. Controls were non-inoculated *E. marginata* stems of the same clonal line (77C40).

Treat-ment	Water or Soil Filtrate	Inoc or Ctrl	Leach for 3 days	Under light for 3 days	Cold shock 30 minutes	Cold shock for 7 days	Bait with leaves	Replicates *n* =
1	SDW	I	+	-	-	-	-	5
1	SF	I	+	-	-	-	-	5
1	SDW	C	+	-	-	-	-	3
1	SF	C	+	-	-	-	-	3
2	SDW	I	+	+	+	-	-	5
2	SF	I	+	+	+	-	-	5
2	SDW	C	+	+	+	-	-	3
2	SF	C	+	+	+	-	-	3
3	SDW	I	+	-	-	+	-	5
3	SF	I	+	-	-	+	-	5
3	SDW	C	+	-	-	+	-	3
3	SF	C	+	-	-	+	-	3
4	SDW	I	+	-	-	-	+	5
4	SF	I	+	-	-	-	+	5
4	SDW	C	+	-	-	-	+	3
4	SF	C	+	-	-	-	+	3

(SDW) = sterile distilled water; (SF) = soil filtrate;
(Inoc) and (I) = inoculated; (Ctrl) and (C) = control.
(+) = treatment imposed; (-) = treatment not imposed.

9a.2.2 *Biological material*

In a field trial (Chapter 5), *E. marginata* stems, of a clonal line (77C40) resistant to *P. cinnamomi* had been inoculated with *P. cinnamomi* isolate MU 94-48. Selected portions of the harvested stems (Fig. 9a.1) were plated onto NARPH selective agar, but no recovery of *P. cinnamomi* was made from some plants though clearly visible surface lesions, characteristic of *P. cinnamomi*, developed on the stems. The remaining portions of these stems, which had been kept at 4±1^{o}C for 7 days were cut into small segments (< 1 cm^{3}) and, after leaching in sterile distilled water (SDW) or soil filtrate (SF), were subjected to further treatment (Table 9a.1) before plating onto the selective

agar. Stem segments of non-inoculated control plants from the field trial, were also used as controls in these experiments.

Soil used for the filtrate was taken from the site of the field experiment (Chapter 5) at Jarrahdale. Prior to immersion of the stem segments, both the SDW and the SF were tested to confirm the absence of *P. cinnamomi* in solution. Leaves of a known host, *Pimelea ferruginea*, were floated in both solutions for 7 days, then plated onto selective agar.

9a.2.3 *Treatments*

Transverse stem sections (< 0.5 cm) were made with a bandsaw, then cut into smaller pieces (< 1 cm x 1 cm x 0.5 cm) with secateurs. Non-sterile soil filtrate was prepared with minesite soil (sieved to remove gravel) in distilled water (25g L^{-1}) then agitated and allowed to stand overnight. It was filtered under vacuum through Whatman's No.1 filter paper. Minesite soil was a mix of soil from 6 locations on the field site from where the plants had been harvested and was tested for the presence of *P. cinnamomi*. Half the pieces were immersed in 2000 ml sterile distilled water and half in 2000 ml soil filtrate. The stem segments were immersed for 3 days and the solutions were replaced daily. By the fourth day, no discolouration of the solutions by phenolic compounds was observed. Solutions in flasks containing pieces of non-inoculated control stems were not discoloured. The stem pieces were then subjected to one of four treatments (Table 9a.1).

In Treatment 1, 5 stem pieces were plated onto each of 5 Petri dishes containing NARPH selective agar.

In Treatment 2, stem pieces were placed under continuous fluorescent light for 3 days, in water or in soil filtrate, at 24±2^{o}C to encourage the formation of sporangia. They were then cold shocked at 4±1^{o}C for 30 minutes to trigger zoospore release before 5 pieces were plated onto each of 5 Petri dishes containing NARPH selective agar. In previous trials (Chapter 3), similar methods (Mehrlich, 1935; Byrt and Grant, 1979; O'Gara, 1998) have successfully produced sporangia and zoospores from mycelial mats of agar when preparing inoculum.

In Treatment 3, stem pieces were placed in 500 ml of SDW or soil filtrate in 5 separate aluminium foil containers in cool conditions at 4±1°C for 7 days, then 5 pieces were plated onto each of 5 plates of NARPH selective agar.

In Treatment 4, stem pieces were placed in 500 ml of SDW or soil filtrate in 5 separate aluminium foil containers. Ten leaves of *Pimelea ferruginea* were floated in all containers to bait any *P. cinnamomi* present. After 7 days, leaves from each container were plated onto NARPH selective agar in separate Petri dishes. The immersed stem segments were plated onto NARPH selective agar. Prior to being plated onto NARPH selective agar, all stem pieces or leaves in each treatment, were blotted dry with absorbent paper, which had been autoclaved at 121°C for 20 minutes on 3 consecutive days.

To test for the presence of *P. cinnamomi* in the SDW or the SF solutions in all treatments, 20 leaves of *Pimelea ferruginea* were floated in 5 separate containers, without stem segments. These functioned as controls for the leaching solutions.

9a.3 Results

9a.3.1 *Recovery of* P. cinnamomi

Recoveries were made from stem pieces in Treatments 1- 4 of the Stem mix. Leaching for 3 days, using water or soil filtrate, then plating onto selective agar was the most successful (Treatment 1, Table 9a.2). No recovery was made from Stem 1 or Stem 2 after being kept at 4±1°C for 7 days (Treatment 3). Baiting *P. cinnamomi* with *Pimelea ferruginea* leaves was more successful when stem pieces were immersed in soil filtrate than in distilled water (Treatment 4). No recoveries were made from control stems, or from *P. ferruginea* leaves in leaching solutions without stem pieces. No results were obtained from the plated stem segments in Treatment 4, because of the accidental disposal of Petri dishes of agar onto which they were plated.

Table 9a.2 Recovery of *P. cinnamomi* from lesioned *E. marginata* stems, after further treatment, when initial direct plating onto NARPH selective agar had indicated no presence of the pathogen in the stems. All plants are of a clonal line of *E. marginata* (77C40) resistant to *P. cinnamomi*.

			No of replicates and recovery (in bracket) of *P. cinnamomi*			
Treatment	Brief description of treatment of stem pieces, prior to plating onto NARPH selective agar	Stem pieces leached in SDW or SF	Stem 1	Stem 2	Four-Stem mix	Total recov-ery
1	Leached for 3 days	SDW	5(5)	5(5)	5(5)	15
1	Leached for 3 days	SF	5(5)	5(5)	5(5)	15
2	Leached, lights, cold shock	SDW	5(2)	5(1)	5(3)	6
2	Leached, lights, cold shock	SF	5(3)	5(2)	5(5)	10
3	Leached, cold treatment	SDW	5(0)	5(0)	5(2)	2
3	Leached, cold treatment	SF	5(0)	5(0)	5(3)	3
4	Baited with leaves	SDW	5(1)	5(1)	5(1)	3
4	Baited with leaves	SF	5(3)	5(2)	5(5)	10

SDW = sterile distilled water; SF = soil filtrate. Leaves = *Pimelea ferruginea* leaves.
Replicate = Petri dish containing stem segments.

9b Growth of *P. cinnamomi* on media amended with exudates from infected stems of *E. marginata* seedlings.

9b.1 Introduction

It was observed that some agar onto which infected *E. marginata* stem segments were plated became darkly stained by the exudates of segments and *P. cinnamomi* was often not recovered from these plates (Chapter 5). Non-infected stems did not stain the agar.

The aim of the current experiment was to determine if the exudates from infected *E. marginata* stems had an effect on the growth and recovery of *P. cinnamomi*.

9b.2 Methods

9b.2.1 *Experimental design*

In two separate experiments, *P. cinnamomi* was grown on NARPH agar (Appendix 3) which had been amended with different concentrations (0%, 25%, 50% and 75%) of exudates leached from the lesioned portions of stems of *E. marginata* seedlings infected with *P. cinnamomi.* The pH of the exudate-amended media was either adjusted to pH 6.0 or not adjusted prior to placing 0.5 cm plugs of actively growing *P. cinnamomi* onto the centre of the plates (Table 9b.1).

Table 9b.1 Protocol for determination of the effect of exudates from infected stems of *E. marginata* seedlings on the mycelial growth *in vitro* of *P. cinnamomi* on selective agar.

Basic medium	Concentration of exudates incorporated into media %	pH not adjusted (n =)	pH adjusted to 6.0 (n =)
NARPH	0	8	20
NARPH	25	8	20
NARPH	50	8	20
NARPH	75	8	20

NARPH = agar selective for *Phytophthora* (Appendix 3).

9b.2.2 *Biological material and preparation of exudate solution*

Lesioned stems of 2-year-old *E. marginata* seedlings, growing in a rehabilitated mine site (Alcoa World Alumina, Australia at Jarrahdale; Alcoa map reference J38 10) and infected in natural conditions with *P. cinnamomi*, were harvested and cut into small segments < $1cm^3$. Infection occurred after heavy summer rainfall provided conditions conducive to an outbreak of the unknown isolate of the pathogen in the soil (Chapter 5). The segments were immersed in sterile distilled water (100g fresh weight L^{-1}) in a conical glass flask. This was agitated several times, then the opening of the flask was covered with a filter paper before being left to stand at room temperature for 3 days. The exudate solution was filtered twice under vacuum, through filter paper (Whatman's No 1). The filtrate was centrifuged at 12000 g for 20 minutes at 15^{o}C. Supernatant was

decanted and centrifuged again as before. Microorganisms were removed from the supernatant using a Millepore 500ml sterile filter unit (Millepore Corporation, Bedford, MA 01730) with a 47 mm diameter membrane (pore size 0.45 μm), attached to a vacuum outlet. The sterile filtrate was added, proportionately, to the NARPH medium. This was repeated in a second experiment where the infected stem material was produced by inoculating 3-year-old *E. marginata* seedlings in the glasshouse with *P. cinnamomi* isolate MU 94-48.

9b.2.3 *Preparation of media*

Sterile exudate solution (pH 5.40 and 5.10) was prepared as above and added to the cooling NARPH medium when the temperature of the NARPH was about 50^{o}C. Concentrations of exudate solution in the media were 0, 25, 50 and 75%. After all components were thoroughly mixed, a sterile syringe dispensed 20 ml of the agar into 90 mm Petri dishes. These plates were incubated at 24^{o}±1^{o}C for 24 hours, to ensure absence of microbial contaminants. Circles, 85 mm diameter, of permeable cellophane (Hallmark Australia Ltd., Caribbean Drive, Scoresby, Vic. 3179), were boiled for 4 hours in deionized water with 2g L^{-1} of ethylenediamine tetraacetic acid (EDTA) added to remove any *P. cinnamomi*-suppressive substances. The cellophane was rinsed, and boiled again in deionized water. It was then autoclaved at 121^{o}C for 20 minutes on 3 consecutive days, before being individually positioned with sterile forceps on the surface of agar in each Petri dish.

9b.2.4 *Preparation of axenic culture of* P. cinnamomi

The *P. cinnamomi* culture was prepared by first passaging an isolate (MU 94-48) of the pathogen through a *E. marginata* seedling and plating segments of the infected stem onto NARPH agar as described in Chapter 3 (Section 3.2.3). The resulting outgrowth of mycelium was sub-cultured onto NARPH until an axenic culture of *P. cinnamomi* was obtained. Plugs of colonized agar, 5mm in diameter, were positioned in the centre of the cellophane discs. Plates were sealed with Parafilm™ and incubated

in the dark at 23±2°C. Diameters of the growing mycelia were measured daily for 7 days.

9b.2.5 *Statistical analysis*

Data were analysed using Statistica® for Windows Version 5 (StatSoft Inc., OK, USA) and Excel® for Windows (Microsoft Corporation, USA), Microsoft Excel Analysis ToolPak (GreyMatter International, Inc., Cambridge, MA 02141). Prior to parametric analyses, data were screened for basic assumptions of parametric tests including normal distribution, equality of variances and non-correlation of means and variances. After comparison of data between or within exudate concentrations, (Table 9b.1), where an ANOVA resulted in a P-value of < 0.05, data were considered significantly different.

9b.3 Results

9b.3.1 *Growth of* P. cinnamomi *on media*

The high (75%) concentration of exudate solution added to the NARPH agar reduced the mycelial growth of *P. cinnamomi* on both media adjusted to pH 6.0 and on media not pH-adjusted (Fig. 9b.1). A two-way ANOVA showed that both pH adjustment and concentration of exudates had a significant ($P<0.001$) effect on mycelial growth. On agar amended with 75% exudate solution, no growth was observed on any of the replicates where the pH was not adjusted, nor on 7 of the 20 replicates where pH was adjusted to 6.0.

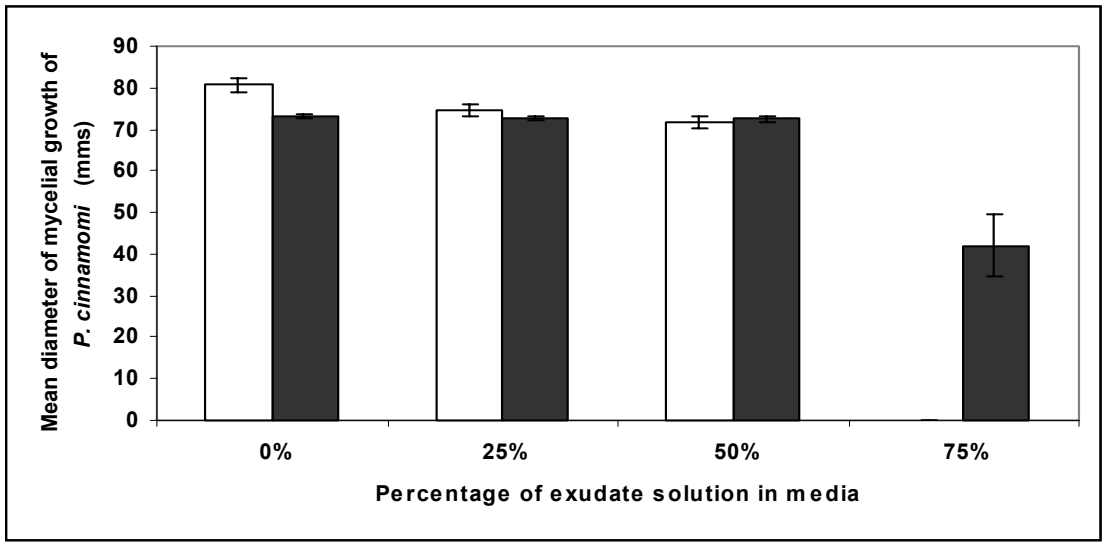


Figure 9b.1 Mean diameters of mycelial growth of *P. cinnamomi* on NARPH agar prepared with increasing percentages of exudate solution. No growth was recorded on agar made with 75% exudate solution when pH was not adjusted. Bars represent the standard error of the mean.

□ pH of media, prepared with exudates of naturally infected stems, not adjusted.

■ pH of media, prepared with exudates of inoculated stems, adjusted to 6.0.

9c Effect of an antioxidant on the *in vitro* growth of *P. cinnamomi*

9c.1 Introduction

It has been shown that exudates, of infected stems of *E. marginata* plated onto selective agar media, can inhibit the growth of *P. cinnamomi* (Chapter 9b) and may mask its presence when standard isolation techniques are employed (Chapter 9a). Exudates include soluble phenolic compounds synthesized by the host as a defence mechanism. Oxidized phenolics can be more toxic to pathogens (Kosuge, 1969; Dimond, 1970) and, in previous harvests (Chapter 5), exudates from *E. marginata* stems have leached onto and stained the selective agar medium. In these cases *P. cinnamomi* was often not recovered. It was reasoned that the addition of an antioxidant to the

medium, onto which infected stems were plated, might counter the effect of the exudates and allow for the recovery of the *P. cinnamomi*.

The aim of this trial was to determine the effect of the antioxidant, ascorbic acid, on the mycelial growth of *P. cinnamomi* on selective agar.

9c.2 Methods

9c.2.1 *Experimental design*

An isolate of *P. cinnamomi* (MU 94-48) was sub-cultured on NAPRH selective agar (Appendix 3), which had been amended with 6 different concentrations of an antioxidant, ascorbic acid. The pH of half the replicates in each concentration was adjusted to 6.0; the remaining half were not pH adjusted (Table 9c.1). Mycelial growth was monitored for 7 days.

Table 9c.1 Protocol for determination of the effect of increasing concentrations of an antioxidant (ascorbic acid) on the mycelial growth of *P. cinnamomi in vitro*.

Basic Medium	Concentration of ascorbic acid mg L^{-1}	Number of Replicates	
		pH not adjusted	pH adjusted to 6.0
NARPH	0	6	6
NARPH	100	6	6
NARPH	500	6	6
NARPH	1000	6	6
NARPH	1500	6	6
NARPH	2000	6	6

NARPH = agar medium selective for *Phytophthora* (Appendix 3).

9c.2.2 *Preparation of media*

NARPH agar was prepared with 6 different concentrations 0, 10, 20 and 30 mg L^{-1}, of ascorbic acid (Sigma Chemical Co., Sigma-Aldrich Pty.Ltd., Castle Hill, NSW, Australia. 2154) added to the cooling agar at about 50^{o}C. The pH of half the amended media of each concentration was adjusted to 6.0. A sterile syringe dispensed 20 ml of the NARPH / ascorbic acid agar to each of the 90 mm Petri dishes. Cellophane discs were prepared (Section 9b.2.3) and placed on the surface of all media. Plates were

incubated at 23±2°C for 24 hours, before the introduction of the *P. cinnamomi*, to check for the presence of any contaminants.

9c.2.3 *Preparation of* P. cinnamomi *culture*

A sub-culture of *P. cinnamomi* was prepared from axenic culture in storage, and plugs of colonized agar, 5mm in diameter, were positioned in the centre of the cellophane discs. Plates were sealed with Parafilm™ and incubated in the dark at 23±2°C. Diameters of the growing mycelia were measured daily for 7 days.

9c.2.4 *Assessment of pH of media*

The volume of media prepared was more than required for the six replicates and 3 extra plates were used to assess the pH of the agar. Indicator paper (Whatman narrow range pH 1-4 and Whatman narrow range pH 4-6; Duotest pH 5-8, Macherey, Nagel and Co. Germany) was inserted between two compressed blocks of agar in the Petri dishes for 5 minutes before colour-matched readings were taken.

9c.2.5 *Statistical analysis*

Data were analysed using Statistica® for Windows Version 5 (StatSoft Inc., OK, USA) and Excel® for Windows (Microsoft Corporation, USA), Microsoft Excel Analysis ToolPak (GreyMatter International, Inc., Cambridge, MA 02141). Prior to parametric analyses, data were screened for basic assumptions of parametric tests including normal distribution, equality of variances and non-correlation of means and variances. After comparison of data between or within concentrations of ascorbic acid (Table 9c.1), where an ANOVA resulted in a P-value of < 0.05, data were considered significantly different.

9c.3 Results

9c.3.1 *pH of media*

As the concentration of ascorbic acid added to the NARPH medium increased, the pH decreased. The pH readings were 6.0, 5.8, 5.8, 4.4, 4.0 and 4.0 in concentrations of 0 mg L^{-1}, 100 mg L^{-1}, 500 mg L^{-1}, 1000 mg L^{-1}, 1500 mg L^{-1} and 2000 mg L^{-1} , respectively.

9c.3.2 *Growth of* P. cinnamomi *on media*

Increased growth of *P. cinnamomi* was observed on all NARPH media amended with ascorbic acid, when the pH was adjusted to 6.0 (Fig. 9c.1). A two-way ANOVA showed that both ascorbic acid concentration and pH adjustment had a significant ($P < 0.05$) effect on mycelial growth of *P. cinnamomi.* There was a strong correlation ($r = 0.92$) between pH and mycelial growth ($n = 72$).

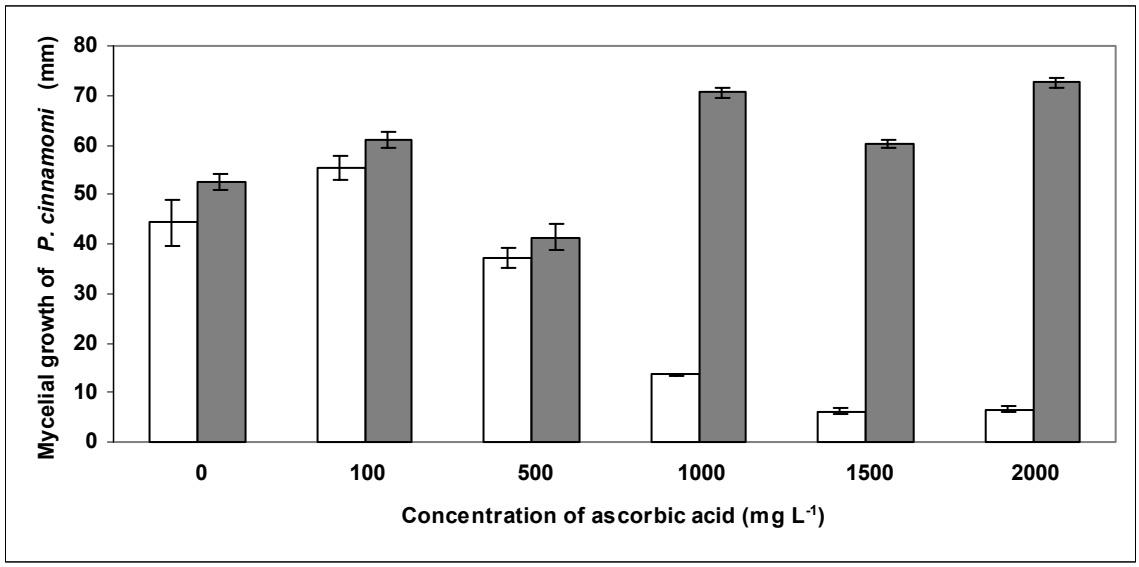


Figure 9c.1 Growth, after 7 days, of *P. cinnamomi* on NARPH agar amended with various concentrations of an antioxidant (ascorbic acid).
Bars represent the standard error of the mean.

□ pH of media was not adjusted ■ pH of media was adjusted to 6.0.

9d The pH effect on the *in vitro* growth of *P. cinnamomi* on media amended with both an antioxidant and with exudates of infected stems of *E. marginata* seedlings.

9d.1 Introduction

Exudates from infected stems of *E. marginata* reduced the growth of *P. cinnamomi* on selective agar, with or without standardized pH (Chapter 9b). The addition of ascorbic acid also lowered the pH of the agar (Chapter 9c) which in turn reduced the growth of *P. cinnamomi* (Chapter 9c and Appendix 7). *P. cinnamomi* is known to grow poorly at pH < 4.0 (Zentmyer, 1980).

The aim of this trial was to examine the interaction of infected stem exudates and an antioxidant, ascorbic acid, when incorporated into selective agar, on the recovery and mycelial growth of *P. cinnamomi*. The pH of the exudate-amended media in half the plates was standardized at pH 6.0. The interaction could more easily be assessed when exudate concentration was constant and the pH was standardized in half the replicates.

9d.2 Methods

9d.2.1 *Experimental design*

An axenic culture of *P. cinnamomi* (isolate MU 94-48) was grown on plates of NARPH selective agar (Appendix 3) incorporating 50% exudate solution from the inoculated stems of *E. marginata* seedlings. Various concentrations of an antioxidant (ascorbic acid) were added. There were 20 replicates of each concentration. The pH of half the plates was standardized to 6.0 (Table 9d.1).

Table 9d.1 Protocol for determination of growth of *P. cinnamomi* on NARPH agar, incorporating 50% exudate solution from inoculated stems of *E. marginata* seedlings. Various concentrations of ascorbic acid were added.

Basic medium	Concentration of ascorbic acid	pH of media not adjusted *n* =	pH of media adjusted to 6.0 *n* =
NARPH with exudates	0 mg L^{-1}	10	10
NARPH with exudates	500 mg L^{-1}	10	10
NARPH with exudates	1000 mg L^{-1}	10	10
NARPH with exudates	1500 mg L^{-1}	10	10

NARPH = agar selective for *Phytophthora* (Appendix 3).

9d.2.2 *Preparation of media*

Sterile exudate solution (pH 5.38) was prepared as described in 9b.2.2 using stems of 3-year-old *E. marginata* seedings grown in the glasshouse. The NARPH selective agar (Appendix 3) was prepared with half the volume of distilled water, augmented with the sterile exudate solution. Eight McCartney bottles containing 10 ml of the distilled water were autoclaved separately at 121^{o}C for 20 minutes on 3 consecutive days. When the water was cool, the ascorbic acid (Sigma Chemical Co., Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia. 2154) was added in concentrations of 0 mg L^{-1}, 500 mg L^{-1}, 1000 mg L^{-1} and 1500 mg L^{-1}. These concentrations were then added to the cooling NARPH agar with the other standard additives and the sterile exudate solution. The pH for half the NARPH and exudate agar, in each concentration of ascorbic acid, was adjusted to 6.0 (Table 9d.1) using 1M NaOH with volumes that had been determined using non-sterile preparations of the mix. The pH of half the media was therefore affected by the addition of the ascorbic acid. Sterile syringes dispensed 20 ml of agar to each 90 mm Petri dish. Extra replicates of all concentrations were prepared.

Discs of permeable cellophane with a diameter of 85mm were boiled for 4 hours in deionized water with 2g L^{-1} of ethylenediamine tetraacetic acid (EDTA) added. They were rinsed, then boiled again in deionized water. They were autoclaved at 121^{o}C for 20 minutes on 3 consecutive days, before being individually positioned with sterile forceps on the agar surface in each Petri dish.

9d.2.3 *Preparation of* P. cinnamomi *culture*

The *P. cinnamomi* culture was prepared by first passaging the pathogen through a seedling of *E. marginata* and plating the infected stem onto NARPH agar. The resulting outgrowth of mycelium was sub-cultured until an axenic culture *of P. cinnamomi* was obtained. Under aseptic conditions, a 5 mm diameter plug of this *P. cinnamomi*–colonized agar as cut with a cork borer and placed in a central position, on the cellophane covering the agar, in each Petri dish. The permeable cellophane allowed the pathogen access to nutrients in the agar but prevented its growth into the agar, forcing the spread of mycelium over the surface of the cellophane where growth could be more accurately monitored. Plates were sealed with Parafilm™ and incubated in the dark at 24±1^{o}C. The diameters of mycelial growth from this plug was measured daily for 7 days.

9d.2.4 *Assessment of pH of media*

After preparation, the pH of media which had not been standardized to 6.0 was assessed with pH indicator paper. Extra replicates of agar for all concentrations of ascorbic acid had been prepared. The indicator paper was inserted between two compressed blocks of agar in the Petri dishes for 5 minutes before colour-matched readings were taken.

9d.2.5 *Statistical analysis*

Data were analysed and graphs produced using Excel® for Windows (Microsoft Corporation, USA), Microsoft Excel Analysis ToolPak (GreyMatter International, Inc., Cambridge, MA 02141).). Prior to parametric analyses, data were screened for basic assumptions of parametric tests including normal distribution, equality of variances and non-correlation of means and variances. After comparison of data between or within concentrations of ascorbic acid (Table 9d.1), where an ANOVA resulted in a P-value of < 0.05, data were considered significantly different.

9d.3 Results

9d.3.1 *pH of media*

The pH of the media decreased with increasing concentrations of ascorbic acid, with readings of 5.5, 4.0, 3.8 and 3.1 in concentrations of 0 mg L^{-1}, 500 mg L^{-1}, 1000 mg L^{-1} and 1500 mg L^{-1}, respectively.

9d.3.2 *Growth of* P. cinnamomi *on media*

The growth of *P. cinnamomi* declined as the concentration of ascorbic acid increased in the media not standardized to a pH of 6.0 (Fig. 9d.1). A two-way ANOVA showed that both pH adjustment and ascorbic acid concentration had a significant ($P < 0.001$) effect on mycelial growth. There was a strong correlation ($r = 0.9$) between pH and mycelial growth at 7 days ($n = 80$).

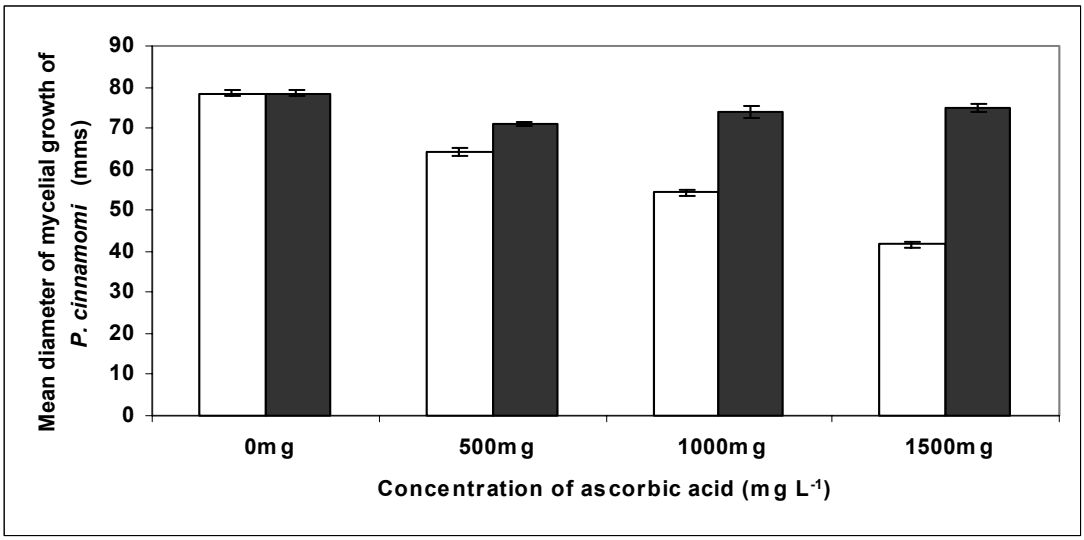


Figure 9d.1 Mean diameter of mycelial growth of *P. cinnamomi* on NAPRH media prepared with 50% exudate solution and amended with various concentrations of ascorbic acid. Bars represent the standard error of the mean.
□ pH of media not adjusted. ■ pH of media adjusted to 6.0.

9.4 Discussion

9.4.1 *The effect of exudates on the growth of* P. cinnamomi

These experiments indicate that exudates from infected *E. marginata* plants can inhibit the growth of *P. cinnamomi* on NARPH, a *Phytophthora*-selective medium, even when the pH is standardized to a level conducive to growth of the pathogen (Chapter 9b). The exudation of inhibitory compounds from plant tissue can therefore result in negative results in the recovery of *P. cinnamomi*. Phenolic compounds have been identified as inhibitory to *Phytophthora* spp. both *in planta* and *in vitro* (Casares *et al*., 1986; Candela *et al.,* 1995). Some phenolics inhibit the *in vitro* growth of *P. cinnamomi* more than others (Cahill and McComb, 1992). Phenolic compounds are a plant's chemical defence against herbivory and disease (Rosenthal, 1986). They are synthesized in *E. marginata* as a defence against leaf miner (Boyce and Bennett, 1996) and are a contributing factor in resistance to *P. cinnamomi* (Cahill *et al*., 1993).

The concentrations of stem exudates incorporated into the media in the current study were diluted in the leaching process (Chapter 9b.2.2) and diluted further before being added to the media. *P. cinnamomi* would encounter undiluted compounds *in planta* which would be expected to have an even greater inhibitory effect than shown here with the diluted exudates. In routine assessment of development of *Phytophthora* disease in plants, stem and root segments are plated onto selective agar. If the growth of *P. cinnamomi* onto agar is directly affected by undiluted inhibitory compounds, the results of disease development may be misinterpreted with falsely negative recovery of *P. cinnamomi*.

The leaching of stem exudates in water or soil filtrate prior to plating onto selective agar, was the most successful treatment used to recover *P. cinnamomi* (Chapter 9a). Recovery was made from all replicates from which the presence of *P. cinnamomi* was not recorded with direct plating. Leaching has increased the percentage of recovery in other studies (O'Gara, 1998; Hüberli *et al*., 2000) and after some harvests (Chapters 5, 6 and 8). However leaching did not always increase the recovery of *P. cinnamomi* (Chapter 5, December and January harvests; Chapter 7).

9.4.2 *The effect of pH on the growth of* P. cinnamomi

The pH of media had a pronounced effect on the mycelial growth of *P. cinnamomi*. In the presence of increasing concentrations of stem exudates growth decreased less on media adjusted to pH 6.0 than on media where pH was not standardized (Chapter 9b). The pH of diluted exudate solutions prepared from infected stems in distilled water (100g L^{-1}) in 9b and 9d was always < 5.5, but within the range (pH 4.0-6.0) most conducive to mycelial growth of *P. cinnamomi* (Zentmyer, 1980). Increasing concentrations of ascorbic acid also lowered the pH of media (Chapter 9c) and resulted in proportionately less growth of *P. cinnamomi*. Where the combination of stem exudates and ascorbic acid lowered the non-standardized pH of media (Chapter 9d), growth was diminished with increasing concentrations of ascorbic acid. However, when the pH was standarized to 6.0 in media with the same components (Chapter 9d), growth was not as severely affected. The pH of undiluted exudates leaching from infected plant tissue onto agar would be expected to significantly lower the pH of media used in direct plating. Levels of pH <3.0 are sub-optimal for, or totally inhibitory to, the mycelial growth of *P. cinnamomi* (Schmitthenner and Canaday, 1983; Zentmyer 1980).

9.4.3 *The effect of an antioxidant on the growth of* P. cinnamomi

Increasing concentrations of the antioxidant, ascorbic acid, in the NARPH selective medium lowered the pH of the agar and reduced *P. cinnamomi* growth (pilot trial, Appendix 7). When this experiment was repeated (Chapter 9c) overall growth was slower on all media irrespective of amendments. This could be because the isolate had not been repassaged prior to sub-culturing on the amended media. An initial increase in growth was noted with a low concentration (100 mg L^{-1}) of ascorbic acid, possibly because of additional nutrients, before rapid decline with increasing concentrations. When the pH of the media was adjusted to 6.0, growth tended to increase with increasing concentrations. Because oxidized phenolics are more toxic to pathogens (Kosuge, 1969; Dimond, 1970), the antioxidant was introduced into the media to counter possible toxic oxidized phenolic compounds from infected stems of *E. marginata*. When the concentration of ascorbic acid was increased, in the presence of stem exudates (Chapter 9d) there was a decrease in the mycelial growth of *P.*

cinnamomi in media where the pH was not standardized to 6.0. The decrease was less than on media with the same concentrations of ascorbic acid but not amended with exudates (Chapter 9c). This could indicate that, in the presence of exudates, the additional nutrients in ascorbic acid had no compensating effect for growth on media with lowered pH levels but was able to support slightly reduced mycelial growth in media at pH 6.0, a level conducive to mycelial growth (Zentmyer, 1980).

When the pH of media with stem exudates and increasing concentrations of ascorbic acid was standardized to 6.0, the mycelial growth was greatest in media with no ascorbic acid but, after an initial decline on a concentration of 500 mg L^{-1}, recovered with increasing concentrations of ascorbic acid. This increase in growth may be due to the increasing proportion of nutrients available to the mycelium, though Chee and Newhook (1965) found that thiamine was the only essential vitamin for mycelial growth of *P. cinnamomi*.

9.4.4 *Other factors affecting the recovery and growth of* P. cinnamomi *on agar*

Leaching for only 3 days without further treatment (Treatment 1, Table 9a.1) led to successful recovery of *P. cinnamomi* from all replicates in both soil filtrate and sterile distilled water. However, where stem segments were leached before further treatment, most recoveries were made from stem segments immersed in soil filtrate to leach the exudates from the plant tissue (Chapter 9a). The continued presence of soil microbes in solution may have had an effect on hyphae, stimulating sporangial formation in the mycelium (Merlich, 1935; Chee and Newhook, 1966; Zentmyer, 1980). In jarrah forest soils, Nesbitt *et al*. (1979a) also found more sporangia in soils with a high proportion of organic matter, but none in sterile soil, indicating that microbial organisms contributed to the formation of sporangia. It is also possible that the release of motile zoospores, from sporangia which formed at the cut surface of the plant tissue, and their subsequent encystment on the agar may have led to the development of faster growing hyphae, which were less affected by any inhibitory compounds still remaining in the tissue.

Temperature is a major factor affecting the growth of *P. cinnamomi*. This has been shown by several researchers (Zentmyer *et al*., 1976; Nesbitt *et al*., 1979b; Phillips and Weste, 1985; Hüberli *et al*., 1997). A series of *in vitro* studies with the same isolate, MU 94-48, used in this thesis, showed that optimal temperatures for growth

were 16°C to 28°C and decreasing temperatures resulted in slower growth (Hüberli *et al*., 1997).

Where possible all growth parameters were kept constant. The presence of cellophane on the agar surface in the experiments allowed a better comparison of radial growth than sub-culturing the pathogen directly onto agar where it could grow down into the agar. *P. cinnamomi* isolate MU 94-48 was used in all experiments where the pathogen was sub-cultured onto agar. It was also used to inoculate all stems used to produce exudate, except for the field-grown seedlings (Chapter 9b) which had been infected by an unknown isolate of *P. cinnamomi*. NARPH agar, selective for *Phytophthora* (Appendix 3) was used throughout.

However, some variables affecting the mycelial growth are unavoidably inconstant. The stem exudates used in these experiments were all obtained from infected *E. marginata* plants, but from different stems at different times. Some were from clonal plants, resistant to *P. cinnamomi* and others from seedlings. The proportion of inhibitory compounds would vary between and within plant types and this factor must be considered in any replication of experiments described here.

While the development of selective agar inhibiting the growth of bacteria and some fungi (Tsao and Ocana, 1969; Masago *et al*., 1977; Tsao and Guy, 1977; Kannewischer and Mitchell, 1981; Jeffers and Martin, 1986; Shearer and Dillon, 1995; Hüberli, 2000) has improved the recovery of *Phytophthora* spp. from infected plants, the presence of *P. cinnamomi* can be masked by faster growing pathogens (e.g. *Fusarium*) in standard direct plating techniques employed in the recovery of *P. cinnamomi* after the harvesting of infected stems if these are present in the host plant.

Interactions between additives in media (e.g. stem exudates and ascorbic acid) and the alteration to these interactions when pH is standardized (Chapter 9d) must be considered. Other interactions are more complex, with not only oxidization of synthesized phenolic compounds and antioxidants but also nutritional levels and pH alteration all contributing to the outcome. Accurate compensation in media preparation cannot be made for inconsistent releases of exudates. However, it is feasible that additives to counter the effect of these exudates can be added to media with pH adjusted to a level that will allow unimpeded growth of the pathogen from the infected stems. Within the same species, it could reasonably be argued that these dynamics would

change with varying abiotic conditions, e.g. incubation temperatures, humidity and light.

9.4.5 Conclusion

Initial recovery figures when assessing the presence or absence of *P. cinnamomi* in a possible host, may be inaccurate or incomplete. It has been shown that immersion in water or soil filtrate, prior to plating onto agar, improves recovery of *P. cinnamomi* from *E. marginata* stems. Further research, trialling other media additives to counter the effect of exudates which impede the growth of *P. cinnamomi* onto selective agar, would be justified. It would be justified because the 'false negatives' (Hüberli *et al*., 2000) distort the results of data, subsequently impacting on management decisions and strategies which attempt to contain the spread of the pathogen in the field.

Chapter 10

The addition of catechol to induce resistance to *Phytophthora cinnamomi* in the roots of *Eucalyptus marginata*.

10.1 Introduction

Phenolic compounds are synthesized *de novo* as part of a plant's defences when wounded or when confronted with invasion by a pathogen (Nicholson and Hammerschmidt, 1992; Bennett and Wallsgrove, 1994). They are known to inhibit, with varying degrees of effectiveness, the *in vitro* growth of *P. cinnamomi* (Casares *et al*., 1986; Cahill and McComb, 1992). Roots of the resistant *Eucalyptus calophylla* (now *Corymbia calophylla*), were found to have higher levels of phenolic compounds than roots of the more susceptible *E. marginata*, prior to infection by *P. cinnamomi*. After inoculation with the pathogen, levels of phenolic compounds increased substantially in *C. calophylla* but little increase was recorded in the *E. marginata* roots (Cahill and McComb, 1992). It has also been shown that exudates of infected stems of *E. marginata* seedlings inhibited the *in vitro* growth of *P. cinnamomi* (Chapter 9).

While biosynthesis of low molecular weight phytoalexins *in planta*, in response to invasion by pathogens, has been recognized (Cruickshank, 1963), investigations into their uptake by roots have been few. A study using susceptible and resistant cultivars of tomato plants and the wilt pathogen *Fusarium oxysporum* f. sp. *lycopersici* found that, after the addition of a phenolic compound, catechol, to the nutrient solution in which the plants were growing, there was an increase in phenolic levels in the roots and the plants displayed a proportionate increase in resistance to the disease caused by *Fusarium* (Retig and Chet, 1974). The current study is the first to investigate the use of catechol in the induction of resistance to *P. cinnamomi* in roots of *E. marginata*. Catechol is a compound which occurs naturally in *E. marginata* tissue (Boyce and Bennett, 1996). If the roots of *E. marginata* seedlings could absorb a phenolic compound in solution, would those supplementary phenolics, in addition to the phenolics synthesized *de novo* after infection with *P. cinnamomi*, be sufficient to increase resistance to the disease?

A hydroponics system, successfully developed in a study with lupin (*Lupinus augustifolius* var. *merrait*) seedlings (Groves, 2000; Appendix 6), incorporated a commercially prepared formulation of catechol with the nutrient solution. The study indicated that low concentrations of catechol (0, 10, 25 and 40 mg L^{-1}) in nutrient solution did not significantly increase the resistance of lupin roots to disease. The concentrations were increased for the experiments with *E. marginata* seedlings.

The aims of Experiment 10a were (1) to re-evaluate and adapt the hydroponics system for *E. marginata* seedlings and (2) to determine the effect that different concentrations of catechol had on the resistance of the seedlings to the disease caused by *P. cinnamomi*.

The aims of Experiment 10b, based on the observations in Experiment 10a, were (1) to improve the methods used and (2) to repeat the experiment with an increased concentration of catechol.

H_o: The addition of a phenolic compound to the nutrient solution in which they are growing, will have no effect on the resistance of *E. marginata* seedlings to the disease caused by *P. cinnamomi*.

10a.2 Methods Experiment a

10a.2.1 *Experimental design*

E. marginata seedlings were grown in a hydroponics system with 3 plants in each of 24 containers of nutrient solution (10a.2.4). Six concentrations, 0, 10, 20, 40, 60 and 80 mg L^{-1} of catechol (Sigma Chemical Company, Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia 2154) were added to the nutrient solution in each of 4 containers. Six seedlings at each catechol concentration were inoculated with *P. cinnamomi* zoospores and the remaining six were sham inoculated with sterile distilled water (SDW) as control plants (Table 10a.1). The level of resistance was assessed by harvesting the seedlings after 5 days, then plating 0.5 cm segments of roots onto agar selective for *Phytophthora* to determine the extent of colonization by the pathogen.

Table 10a.1 Protocol for inoculation, with *P. cinnamomi,* on roots of *E. marginata* seedlings growing in nutrient solution to which different concentrations of catechol were added. The 6 replicates in each treatment were separated into 2 containers.

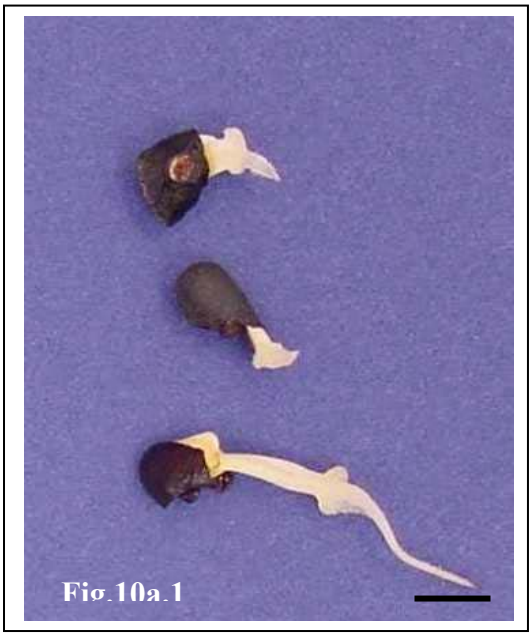
Treatment	Inoculated with zoospores	Catechol concentration mg/L	Replicates *n* =
1	+	0	6
2	-	0	6
3	+	10	6
4	-	10	6
5	+	20	6
6	-	20	6
7	+	40	6
8	-	40	6
9	+	60	6
10	-	60	6
11	+	80	6
12	-	80	6

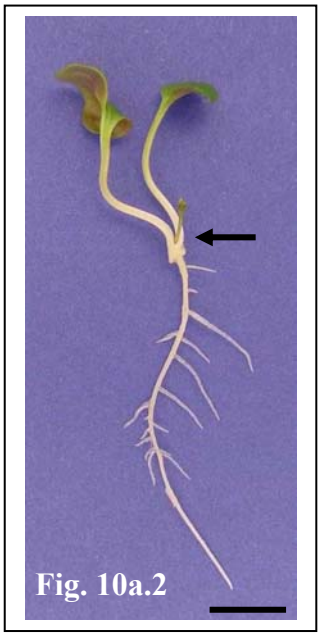
10a.2.2 *Seed germination and early growth*

E. marginata seeds from a seed mix (provenance Nanga, Alcoa Zone 5) obtained from Alcoa World Alumina, Australia were pre-treated and germinated *in vitro* (Appendix 8). Seeds were considered to have germinated when the radicle was visible (Fig.10a.1). Cotyledons appeared and after 21 days true leaves were beginning to form (Fig. 10a.2). When cotyledons appeared, seedlings were transferred to 150mm Petri dishes on 125mm filter paper (Whatman's No.1). Ten seedlings of similar size and age were positioned across the diameter of the base filter paper in the Petri dish. The developing root system was protected from light by folding another filter paper in half and covering the roots while the cotyledons emerged above (Fig. 10a.3). Sterile distilled water kept the filter papers wet and sealing the Petri dishes minimized loss of moisture. The Petri dishes were stacked at an 80^{o} angle under fluorescent light (40μE m^{2} sec^{-1}) at 24^{o}C for 14 days before 72 seedlings were transferred to the hydroponics system.

10a.2.3 *Hydroponics equipment*

To maintain clean growing conditions, distilled water, materials and equipment were autoclaved at 121^{o}C for 20 minutes on 3 consecutive days. Some materials were surface sterilized with 70% ethanol, then irradiated under ultra-violet light for 16 hours prior to use. Twenty-four 2L plastic containers (16.5 x 16.5 cm)





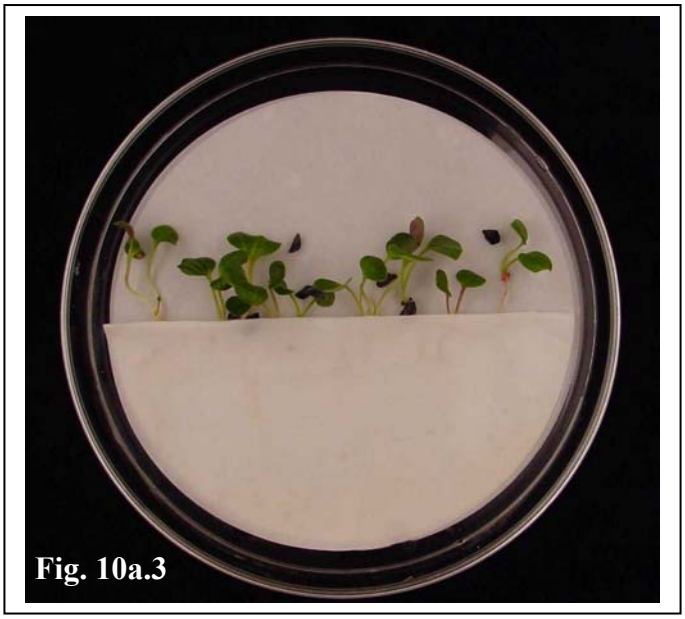


Figure 10a.1 Radicles emerging from *E. marginata* seeds. Scale bar = 5 mm.

Figure 10a.2 Twenty-one-day old *E. marginata* seedling with cotyledons. True leaf (arrowed) is forming at the shoot apex. Scale bar = 1 cm.

Figure 10a.3 Twenty-one-day old *E. marginata* seedlings growing in a 150 mm Petri dish on wet filter paper, with roots protected from light**.**

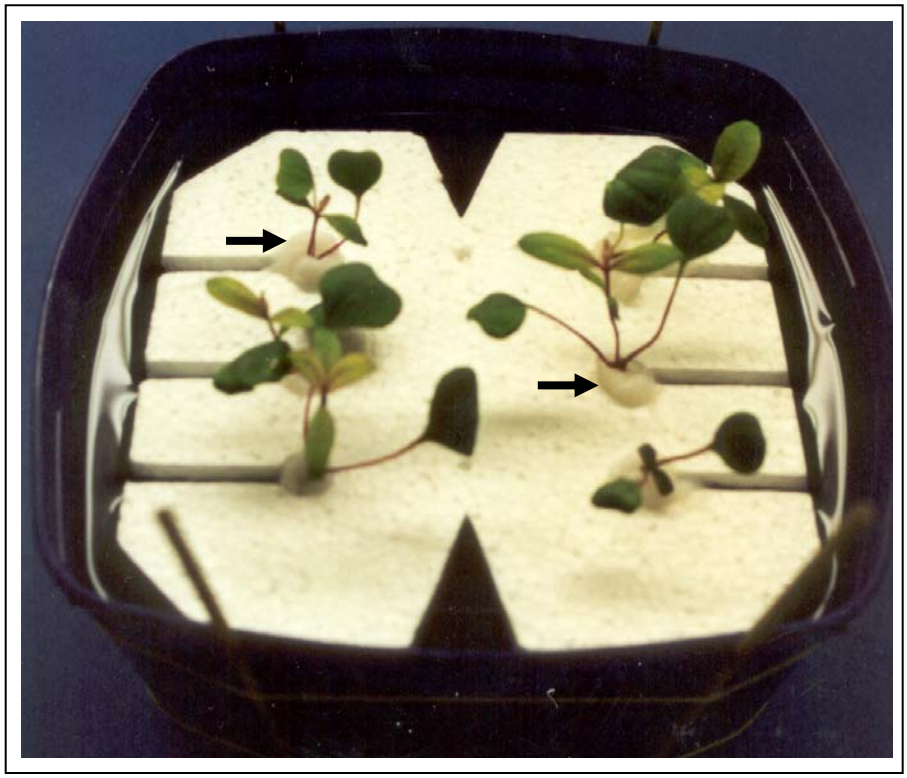


Figure 10a.4 Six-week-old *E. marginata* seedlings, supported by a polystyrene raft, growing in a container (16.5 x 16.5 cm) of nutrient solution. Seedlings were later reduced to three per container. Cotton wool (arrowed) protected and supported the seedlings.

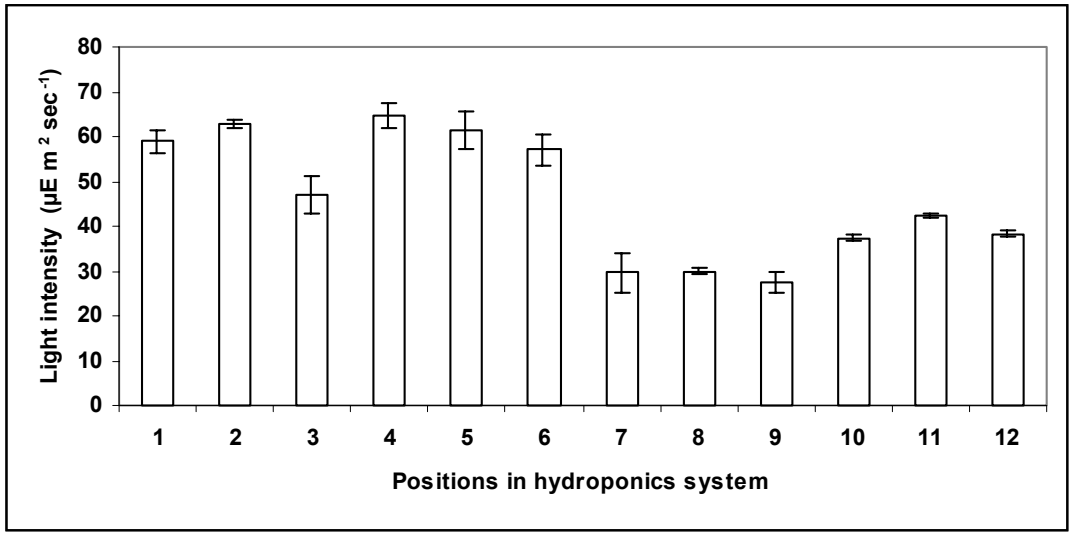


Figure 10a.5 Light measurements (µE m^2 sec^{-1}) at 6 positions above containers on each of the 2 shelves in the hydroponics system. Positions 1-6 were measured on the top shelf and positions 7-12 on the lower shelf. Bars represent the standard error of the mean.

were filled with 1.5L nutrient solution. Three seedlings were inserted into holes in a purpose-designed polystyrene raft, which floated on the surface of each container (Fig. 10a.4). This allowed seedlings to be slid into place without damage to the roots. A protective wrap of cotton wool between the stem and the polystyrene secured each seedling. Tubing with a diameter of 0.5 cm connected all containers to an external air supply and kept the nutrient solution in each container aerated as seedlings grew under fluorescent lights. The amount of light was read 3 times at 6 random points on the 2 shelves of the hydroponics system, using a light meter (Li-Cor, LI-1850), and because of varied results (Fig. 10a.5), containers were moved at random to a different position after each weekly change of nutrient solution. Aquarium airstones were attached to the ends of the hoses in all containers to disperse the incoming air in a way that would not disturb the roots of the seedlings. Blockages in some airstones interrupted the flow of air. They were discarded and the rate of flow to each container adjusted to provide a supply of air (non-sterile), through open-ended hoses, which would aerate the solution without excessive agitation.

10a.2.4 *Nutrient solution*

The nutrient solution was a modification of Hoagland's solution (Hoagland and Arnon (1950), cited by Kannewischer and Mitchell, 1981). A stock solution of micronutrients was made and 1 ml L^{-1} of the stock solution added to the macronutrient solution. Iron in the form of FeNaEDTA, was then added at the rate of 10 mg per litre (Table 10a.2). The nutrient solution was discarded weekly and replaced with freshly made nutrient solution in containers surface sterilized with 70% ethanol. Seedlings were repositioned in duplicate replacement rafts, which had been UV-irradiated overnight to sterilize surfaces.

Table 10a.2 Components of nutrient solution used in hydroponics system (modified from Hoagland and Arnon, 1950).

Macronutrients per litre of sterile distilled water:		
Potassium phosphate	KH_2PO_4	68.04 mg
Magnesium sulphate	MgSO4.7H2O	123.02 mg
Potassium nitrate	KNO3	176.75 mg
Calcium nitrate	$Ca(NO_2)_2.4H_2O$	472.00 mg
Micronutrients per litre of sterile distilled water:		
Sodium	$Na_2\ MoO_4.2H_2O$	0.012g
Copper sulphate	$CuSO_4.5H_2O$	0.065g
Manganese	MnSO4.7H2O	0.692g
Boric acid	H_3BO_3	0.773g
Zinc sulphate	$ZnSO_4.5H_2O$	1.006g
Iron	FeNaEDTA	0.010g

10a.2.5 *Addition of phenolic compound*

Six different amounts of catechol, 0, 10, 20, 40, 60, 80 mg L^{-1}, were added to the nutrient solution in containers (Table 10a.1). Each concentration was held in 4 replicate containers, 2 with plants to be inoculated and 2 with control plants. Roots of seedlings were immersed in the nutrient solution with catechol additive for 3 days, prior to inoculation.

10a.2.6 *Inoculation*

Seedlings were inoculated on the root tip with zoospores of *P. cinnamomi*, isolate MU 94-48. Zoospores were prepared as described in Appendix 5. Motile zoospores in suspension were diluted with SDW in an acid washed beaker to approximately 4000 zoospores ml^{-1}. Seedlings were removed from the rafts in the containers and the roots were sprayed with SDW to clean the epidermal tissue and to remove traces of nutrient solution and catechol. Seedlings to be inoculated were placed in Petri dishes. A pipette dispensed 5µl of the zoospore suspension to each root tip. The root tips of control plants were sham-inoculated with 5µl of SDW. After inoculation, seedlings remained in the Petri dish for 1 hour and roots were lightly covered with GLAD® Wrap (Union Carbide Australia Ltd., Sydney NSW. 2001). This allowed time for encystment of the zoospores and for the infection process to begin at the point of inoculation. If returned immediately to the nutrient

solution, motile zoospores could disperse through the solution and infect the roots at points other than the tip. The extent of colonization in individual seedlings would then not be standardized. The exposed shoots of all seedlings were mist-sprayed with SDW to minimize desiccation. Seedlings were returned to catechol-free nutrient solution in the hydroponics system. Replacement containers and rafts were surface sterilized as before (10a.2.4).

10a.2.7 *Harvest*

All seedlings were harvested 5 days after inoculation when many were showing signs of lesion development or had softened, water soaked regions on the root. The detection of necrotic tissue was made more difficult when some roots were darkened by the higher concentrations of catechol. Seedlings were removed from the nutrient solution and laid on glass. The roots were cut, with a sterile scalpel, from 10 cm above the inoculated tip and down to the tip, in 0.5 cm segments. These were transferred to 90 mm Petri dishes of NARPH (Appendix 3), an agar medium selective for *Phytophthora* (Hüberli *et al*., 2000). The plates were sealed with Parafilm™, incubated at 24±1^{o}C and monitored for 14 days for the presence of *P. cinnamomi*.

10a.2.8 *Statistical analysis*

Data were analysed using Statistica® for Windows Version 5 (StatSoft Inc., OK, USA) and Excel for Windows (Microsoft Corporation, USA), Microsoft Excel Analysis ToolPak (GreyMatter International, Inc., Cambridge, MA 02141). Prior to parametric analyses, data were screened for basic assumptions of parametric tests including normal distribution, equality of variances and non-correlation of means and variances. After comparison of data between concentrations of catechol (Table 10a.1), where an ANOVA resulted in a P-value of < 0.05, data were considered significantly different.

10a.3 Results Experiment a

10a.3.1 *Seed germination and growing conditions*

After seeds germinated in the dark (Appendix 8), seedlings developed healthy green cotyledons and true leaves when transferred to 135 mm Petri dishes under fluorescent light at 24^{o}C and grew well with roots in SDW without added nutrients for 14 days. At that time some leaves were becoming chlorotic and some roots were adhering to the filter paper. These problems were addressed in Experiment 10b.2.2.

10a.3.2 *Recovery of* P. cinnamomi

Most recoveries of *P. cinnamomi* were made from roots immersed in the lower concentrations of catechol prior to inoculation (Table 10a.3). No recoveries were made from sham-inoculated control plants.

Table 10a.3 Recovery of *P. cinnamomi* from inoculated roots of *E. marginata* seedlings grown in different catechol concentrations.

Catechol concentration mg L^{-1}	Roots inoculated (n =)	Recovery of *P. cinnamomi* from inoculated roots (n =)
0	6	6
10	6	6
20	6	6
40	6	6
60	6	5
80	6	3

10a.3.3 *Colonization of roots*

There was a significant (*df* 5, 30; P = 0.001) difference between treatments in the extent of *P. cinnamomi* colonization of the *E. marginata* roots. Least development of the disease was in roots growing in solutions with a catechol concentration of 60mg L^{-1} and 80mg L^{-1} (Fig. 10a.6). Prior to inoculation, roots immersed in the two highest concentrations of catechol remained intensely discoloured after washing. Roots in nutrient solution without catechol were not discoloured.

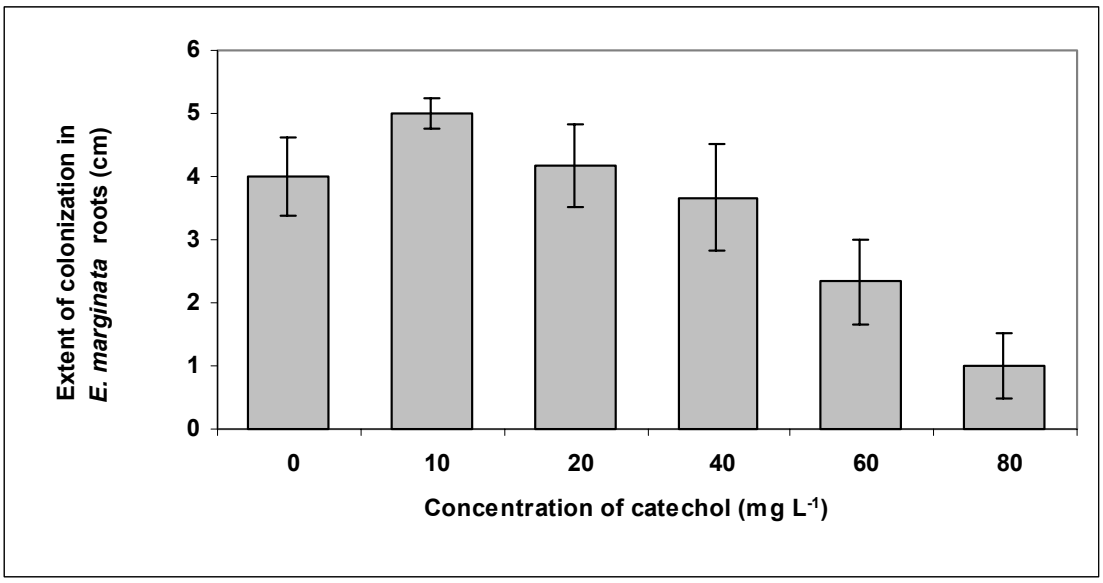


Figure 10a.6 Mean colonization of roots of *E. marginata* seedlings inoculated with *P. cinnamomi* and growing in nutrient solution with different concentrations of catechol additive. Bars represent the standard error of the mean.

Table 10a.2 One-way ANOVA of total colonization of roots immersed in catechol solution.

Catechol Conc. mgL-1	*Count*	*Sum*	*Average*	*Variance*
0	6	24	4.00	2.40
10	6	30	5.00	0.40
20	6	25	4.17	2.57
40	6	22	3.67	4.27
60	6	14	2.33	2.67
80	6	6	1.00	1.60

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	62.81	5	12.56	5.4221	0.0011	2.5336
Within Groups	69.50	30	2.32			
Total	132.31	35				

10b.2 Methods Experiment b

The design of Experiment 10b and some of the methods were modified after the completion of Experiment 10a. That experiment and the pilot trial with lupin seedlings (Appendix 6) both showed that low levels of catechol had little effect on colonization of the roots by *P. cinnamomi*. In Experiment 10b, levels of catechol concentration were increased.

10b.2.1 *Experimental design*

An algal outbreak in the hydroponic containers disrupted this experiment, contaminating seedling roots during the growing stage. This left twenty unaffected seedlings and the experiment was redesigned with fewer replicates. In a hydroponics system, roots of 20 three to four-month-old *E. marginata* seedlings were immersed in 4 different concentrations of catechol. The roots were inoculated with zoospores of *P. cinnamomi* or sham inoculated with sterile distilled water (SDW) and harvested 5 days later for assessment of colonization of the root by *P. cinnamomi* (Table 10b.1). There were 20 containers, 5 of each concentration of catechol, placed at random in the hydroponics system, with 1 seedling in each.

Table 10b.1 Protocol for the assessment of *P. cinnamomi* colonization of *E. marginata* roots after immersion in different concentrations of catechol in nutrient solution in a hydroponics system.

Concentration of catechol mg L^{-1}	Inoculation of roots with *P. cinnamomi* zoospores	Replicates *n* =
0	+	3
0	-	2
60	+	3
60	-	2
80	+	3
80	-	2
100	+	3
100	-	2

\+ = inoculated with zoospores
\- = sham-inoculated with sterile distilled water.

10b.2.2 *Seed germination and growth*

E. marginata seeds, from the same seed mix used in Experiment 10a, were treated and monitored as described in Chapter 10a.2.2 except for 2 changes. Discs of permeable cellophane (Hallmark Australia Ltd., Caribbean Drive, Scoresby, Vic. 3179), 125 mm diameter were boiled for 4 hours in deionized water with 2g L^{-1} of ethylenediamine tetraacetic acid (EDTA) added. After rinsing, they were boiled again in deionized water. Filter papers, 125 mm diameter, Whatman's No.1, and the cellophane discs, were autoclaved at 121^{o}C for 20 minutes on 3 consecutive days. When seedlings were transferred to 150mm Petri dishes, on a base of filter paper, the lower half of the filter paper was covered by a semicircle of permeable paper cellophane. The roots were protected with another overlaying semicircle of cellophane and another filter paper, folded in half to prevent the developing roots adhering to the filter paper, which was a problem in Experiment 10a, while still allowing them access to nutrients and providing protection from the light. When seedlings developed 2 leaves beyond the cotyledon stage, the SDW was replaced by 20 ml of nutrient solution in the Petri dish for 14 days.

10b.2.3 *Hydroponics equipment*

The equipment used in the hydroponics system was as described in Chapter 10a.2.3 except that after seedlings were transferred from the Petri dishes into the containers, they were enclosed in plastic bags to maintain humid conditions, similar to those of the Petri dish environment, for the first 3 days after the transfer (Fig. 10b.1). Small holes made daily in the bags reduced the humidity gradually, until bags were removed after 14 days.

10b.2.4 *Nutrient Solution*

A modified version of Hoagland's nutrient solution (10a.2.4) was used.

10b.2.5 *Algal outbreak and trial redesign*

An outbreak of algae (*Chlorella* sp.) contaminated the nutrient solution in some containers, 7 days after removal of the plastic bags. All equipment was autoclaved again or surface sterilized with a solution of 70% ethanol and placed

under ultra-violet light for 14 hours before nutrient solution was replaced. Opaque black plastic was wrapped around the containers to prevent light penetration and subsequent algal growth. A square was cut in the top on which the polystyrene raft could rest. Only the uncontaminated seedlings were used in the experiment in the replicate numbers shown (Table 10b.1).

.

10b.2.6 *Catechol concentrations*

Four concentrations of catechol (Sigma Chemical Company, Sigma-Aldrich Pty.Ltd., Castle Hill, NSW, Australia) 0, 60, 80 and 100 mg L^{-1} were introduced into the nutrient solution with the 12-week-old seedlings. Roots of seedlings were suspended in the catechol/nutrient solution for 72 hours until inoculation.

10b.2.7 *Inoculation*

Seedlings were laid on the base of individual sterile 150mm Petri dishes and inoculated as described in Section 10a.2.6.

10b.2.8 *Incubation conditions*

After inoculation, seedlings were not returned to the containers in the hydroponics system, because of the potential threat of another algal outbreak. Instead, each seedling was kept under light in an individual 135mm Petri dish, with filter paper and cellophane (10b.2.2), and to each of which 20ml of nutrient solution was added. After 5 days, seedlings were harvested.

10b.2.9 *Harvest*

Lesions were not readily visible on the roots, which had been darkened by the catechol solution. In contrast, there were discoloured, softened regions in the inoculated roots growing in nutrient solution containing no catechol. At harvest, root lengths were noted and roots were cut from 10 cm above the root tip and in 1 cm segments down to it (Fig. 10b.2). the segments were plated onto NARPH selective agar (Hüberli *et al*., 2000) to be assessed for the extent of colonization of the roots by *P. cinnamomi* (Table 10b.1). Plates were sealed with Parafilm™ and incubated in the dark at 24±1^{o}C. Growth of *P. cinnamomi* mycelium on agar was monitored for 10 days.

Figure 10b.1 *E. marginata* seedlings were transferred from a Petri dish to a container (16.5 x 16.5 cm) of nutrient solution and enclosed in a plastic bag to retain humidity for the first three days in the hydroponics system. Wooden skewers were sufficient to retain the air space needed.

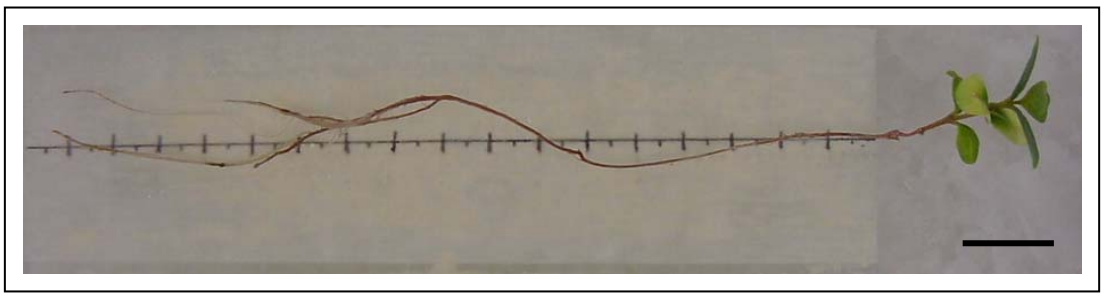


Figure 10b.2 Preparing the root of an *E. marginata* seedling for harvest. Ten x 1 cm segments were taken from above the root tip and down to it, then plated onto agar selective for *Phytophthora*. Scale bar = 2 cm.

10b.2.10 *Statistical analysis*

Data were analysed using Statistica® Version 5.0 (StatSoft Inc., OK, USA) and Excel for Windows (Microsoft Corporation, USA; Microsoft Excel Analysis ToolPak (GreyMatter International, Inc., Cambridge, MA 02141). Prior to parametric analyses, data were screened for basic assumptions of parametric tests including normal distribution, equality of variances and non-correlation of means and variances. In cases where data did not conform to assumptions, log transformations were used to correct the deviations. The use of such transformations is noted in the relevant results. After comparison of data between concentrations of catechol (Table 10b.1), where an ANOVA resulted in a P-value of < 0.05, data were considered significantly different.

10b.3 Results Experiment b

10b.3.1 *Seed germination*

Seeds were considered to have germinated when the radicle was visible. After 28 days, 62% of all *E. marginata* seeds prepared for Experiment 10b had germinated and seeds continued to germinate after this time.

10b.3.2 *Root lengths*

Longest root length was seen in the nutrient solution with no catechol added (mean 19.66±7.67 cm), but a one-way ANOVA of log-transformed root lengths showed no significant (*df* 3, 8; $P > 0.05$) difference between growth in different concentrations of catechol.

10b.3.3 *Recovery of* P. cinnamomi *from root*

Recoveries of *P. cinnamomi* were made from the inoculated roots of all 3 replicates in the 0 mg L^{-1} and 60 L^{-1} mg concentration of catechol. Recoveries were made from only 2 of the 3 replicates in the higher concentrations of 80 mg L^{-1} and 100 mg L^{-1}. No recoveries were made from roots of any sham-inoculated control seedlings

10b.3.4 *Colonization of roots*

There was a reduction in the mean extent of colonization of the roots in higher levels (80 and 100mgL^{-1}) of catechol concentration (Fig. 10b.3). A single factor ANOVA showed no significant (*df* 3,8; P >0.05) difference in colonization between catechol concentrations. There was no strong correlation between root length and colonization of root ($r = 0.3$).

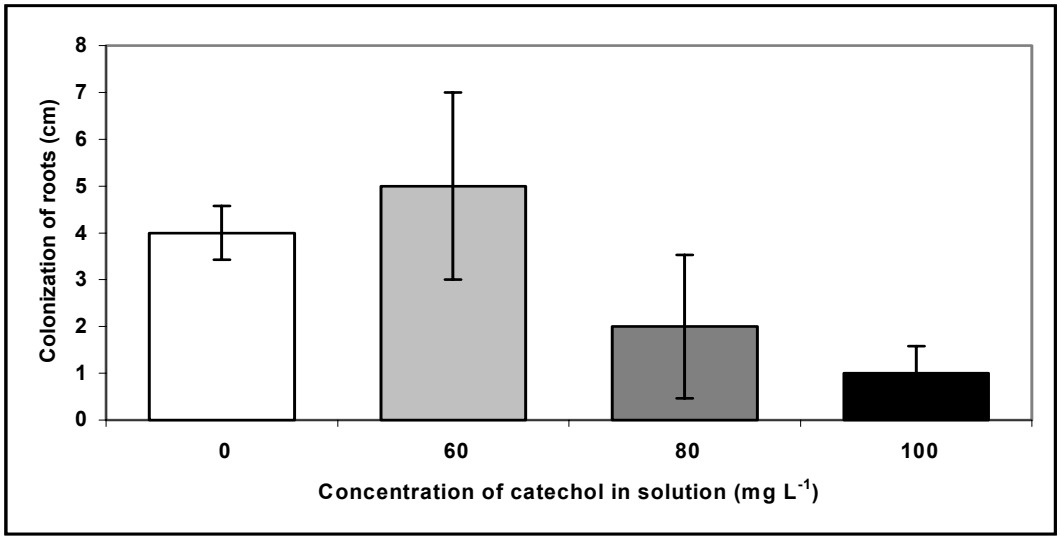


Figure 10b.3 Mean colonization of roots of *E. marginata* seedlings inoculated with *P. cinnamomi* and growing in nutrient solution with different concentrations of catechol additive. Bars represent the standard error of the mean.

10b.3.5 *Improved methods*

Repetition of the experiment was of benefit, allowing problems previously encountered to be addressed. Increased levels of catechol proved to be more effective than low levels. The adhesion of roots to filter paper was overcome by the insertion of a cellophane layer. Seedling shock when transferring from sealed Petri dish to open hydroponics system was reduced by enclosing containers in plastic bags and gradually reducing humidity. Greater protection from possible algal contamination was given when containers were enclosed in opaque covers. After inoculation, seedlings were kept in individual Petri dishes rather than returned to the hydroponics system. This avoided the possibility of algal contamination and in experiments with larger numbers of replicates would avoid pseudoreplication. In addition, observations were made of root lengths at harvest.

10.4 Discussion

10.4.1 *Catechol levels and colonization by* P. cinnamomi

Increased levels of catechol added to the nutrient solution prior to inoculation reduced the extent of colonization by *P. cinnamomi* in roots of *E. marginata* seedlings growing in the hydroponics system.

Since catechol was not present in the solution after inoculation, this trend would indicate an increased resistance to the disease caused by *P. cinnamomi* when roots were pre-treated with the added phenolic compound prior to inoculation. The trend was most noticeable in the two highest concentrations, 60 mg and 80 mg L^{-1} in Experiment 10a and 80 mg and 100 mg L^{-1} in Experiment 10b. In the pilot trial using lupins (Appendix 6; Groves, 2000), it was shown that 40 mgL^{-1} catechol was insufficient to suppress *P. cinnamomi*. Lesion length was impossible to measure due to root discolouration. The persistence of discolouration after washing suggests residual catechol on the epidermal layer. Adhering catechol may be a factor in the decrease in colonization of roots immersed in higher levels of catechol, as it may have inhibited the encystment of zoospores or penetration of root tissue by germinating zoospores. Alternatively, the increased resistance shown in both experiments may be due to an uptake of catechol by the root tissue. The low molecular weight of this compound would possibly enable it to get across the plasmalemma.

Suppression of disease symptoms caused by *Fusarium oxysporum* f. sp. *lycopersici* has been observed in tomato plants when pre-treated with catechol in solution (Retig and Chet, 1974). They suggested that catechol inhibited fungal pathogenesis without inhibiting fungal growth. However, in the current experiments, fewer recoveries of *P. cinnamomi* from the inoculated roots and less colonization of the *E. marginata* seedlings which were pre-treated with higher concentrations of catechol, indicates not only inhibition of pathogenesis but also inhibition of growth of the pathogen *in planta*.

10.4.2 *Root growth*

Root growth was less in seedlings immersed in catechol, indicating that catechol suppressed root extension, but this needs to be investigated further with more replicates. If similar results were obtained, the benefits of increased resistance may be countered by less nutrient uptake and less shoot growth. However, this may be a temporary condition which would be overcome when seedlings adapt to a soil substrate. After planting, the newly formed tissue of growing roots would not be protected if the increased resistance is only the result of catechol adhering to the root surface, but if both adhesion and uptake of catechol are involved, then resistance may be effective for some time as roots grow.

10.4.3 *Conclusion*

The consistent reduction of colonization by *P. cinnamomi* in both these experiments justify further research into the effect of root immersion in catechol (or other phenolic compounds). Future experiments should have more replicates than were used here and should also investigate for how long the inhibition is effective. This may help to resolve whether the effect of catechol is because of uptake by the roots or because of adhesion to the root surface. Modified Folin-Ciocalteau assays (Lowry, 1951; Moerschbacher *et al*., 1988) could be conducted to determine the levels of phenolic compounds and HPLC could detect catechol levels in different root tissue.

Spraying susceptible plants with phosphite to increase resistance to the disease caused by *P. cinnamomi* has become a common practice (Hardy *et al*., 2001) but alternative strategies to phosphite are required as there is evidence of *P. cinnamomi* developing resistance to this fungicide (Dobrowolski *et al*., 2003).

More questions need to be answered.

- Would continued immersion of roots in catechol (or a solution of another phenolic compound) increase resistance to disease?
- Could this pre-treatment be applied to seedings which are intended for planting in revegetation of possible *P. cinnamomi* infected soil, e.g. in mine sites?

- How does the cost of pre-treatment before planting of susceptible plants (immersion of roots and stem in a solution of phenolic compounds or spraying of plants with a solution of phenolic compounds) compare to the cost of spraying with phosphite or similar fungicides?
- If roots do not uptake phenolic compounds but are only protected when catechol adheres to the epidermal surface, can the effect be sustained with or without repeated treatment to provide an alternative method of defence against *P. cinnamomi*?

Continued research can answer these questions. It is possible that treatment of roots with phenolic compounds will improve the rate of seedling establishment in soil where there is a potential threat of *P. cinnamomi* and prove to be a useful adjunct to phosphite treatment.

Chapter 11
General Discussion

11.1 Overview

The pivotal study in this PhD thesis was the field trial (Chapter 5), for which a new inoculation technique for *Phytophthora cinnamomi* was developed, and which provided new directions in the research. On a rehabilitated bauxite mine site, resistant *Eucalyptus marginata* plants were inoculated with *P. cinnamomi* (Chapter 5) using the new non-wounding technique (Chapter 4). The new method allowed a more realistic pathogenesis than did conventional wounding techniques and was successfully applied both in the field (Chapter 5) and in subsequent glasshouse experiments (Chapters 6, 7 and 8). It overcame the logistical problems of the non-wounding zoospore inoculation technique and is a major step forward in the study of *P. cinnamomi*, particularly relevant to the study of *P. cinnamomi* infection in ponded riplines of rehabilitated mine sites.

This thesis has repeatedly shown that the availability of water is a major factor in the development of disease caused by *P. cinnamomi* in *E. marginata*. The pathogen colonized well-watered plants which experienced no water deficit more extensively than those which were droughted. It showed conclusively that the established maxim that many pathogens are more aggressive when the host is subjected to water deficit (Schoeneweiss, 1975; Boyer, 1995), does not apply to this host/pathogen interaction. The current study supported previous findings that colonization of *E. marginata* by *P. cinnamomi* was less when bark moisture and plant water status (Tippett and Hill 1983; Tippett *et al*., 1987) or rainfall (Bunny *et al*., 1995) was lower. The study also extends the comparison between different watering regimes to different inoculation methods, genotypes and the timing of inoculation and harvests.

The heavy summer rainfall event invalidated the original experimental design of the field trial, which was intended to monitor and compare disease development in droughted plants and in irrigated plants (Chapter 5). Instead, it unexpectedly presented a unique opportunity to observe the natural spread of disease which was exacerbated by this rare event. This is the first study to monitor concurrently, in a

rehabilitated mine site after heavy summer rainfall, (1) the natural spread of *P. cinnamomi* and (2) the effect of the pathogen on genetically resistant clonal plants and on seedlings of *E. marginata*.

Recovery of the pathogen from symptomless tissue and non-recovery from lesioned stems (Chapter 5) led to a series of studies which clearly showed the inhibitory effect of exudates from infected stems on the *in vitro* growth of *P. cinnamomi* and this led to an investigation of methods to improve the recovery of *P. cinnamomi* from infected stems (Chapter 9). Finally, a novel approach to improve the resistance of *E. marginata* to *P. cinnamomi* was explored (Chapter 10). The introduction of a range of concentrations of a phenolic compound, catechol, to the nutrient solution in which *E. marginata* seedlings grew, resulted in less colonization of roots with higher concentrations of catechol, indicating an improved level of resistance. These results and other results in the thesis raise interesting questions that can lead to further research.

11.2 Inoculation with *P. cinnamomi*

The need for an effective, non-invasive method of inoculation for woody plants was apparent after the rapid deaths of *E. marginata* seedlings wound-inoculated with *P. cinnamomi* using the underbark method (Chapter 3). In the same experiment, a concurrent evaluation was made of the non-wounding zoospore inoculation method, developed to simulate the infection court presented by ponded riplines in mine sites (O'Gara *et al*., 1996). After an evaluation of the time spent and the logistical problems presented by the method of O'Gara *et al*. (1996), the new non-wounding inoculation method was developed. Stems were pre-treated with wet cotton wool and mycelial plugs applied to the moistened area. Replacement wet cotton wool, simplified the simulation of ponding and resulted in highly successful stem infection, without wounding (Chapter 4). The conceptual design of this technique was influenced by the frequent observations of stem infection in *E. marginata* in ponded riplines in rehabilitated mine sites (Dr G. Hardy and Dr I. Colquhoun, *pers. comm*.). It was successfully applied in the field trial with 73% recovery of *P. cinnamomi* from inoculated *E. marginata* plants, resistant to the pathogen (Chapter 5). It was also successful in glasshouse experiments (Chapters 6,

7 and 8) and has since been used by other researchers investigating the interactions between other hosts and pathogens, including *E. globulus* with *Endothiella* (T. Jackson, *pers. comm*.), *Grevillea* spp. with *P. cinnamomi* (Dr. M. Dobrowolski, *pers. comm*.) and *Banksia grandis* with *P. cinnamomi* (Auckland, 2002). Previously, forest pathologists had used inoculation techniques which required mechanical wounding of the host before the placement of inoculum in direct contact with the phloem (Tippett *et al*., 1983; Shearer *et al*., 1987; Davison *et al*., 1994). The introduction of inoculum directly to the sugar-rich phloem and the limited time for host defence responses to be activated, gives the pathogen an abnormal advantage (Chapter 3). Wounding methods are effective and useful for some observations but represent neither a realistic interaction between host and pathogen nor a typical infection process. Though *P. cinnamomi* may opportunistically invade wounded hosts in a natural situation, O'Gara *et al*. (1996) found that it can also infect non-wounded tissue. The success of the non-wounding technique in the current study (Chapter 4) supports that finding, and is important because the timing of plant defence responses to pathogen attack and/or wounding must be considered and is further discussed in Section 11.3. It is particularly relevant to pathogenesis in rehabilitated mine sites in Western Australia where currently over 550 hectares of jarrah forest are mined and rehabilitated annually. Though a significant portion of this area is free of the pathogen, some parts are adjacent to infested sites. Ponds of water, retained by the fine clay sediment washed into riplines in the mine sites, provide an infection court for the zoospores of *P. cinnamomi*. In this microenvironment, it is not the roots of the plants, but the collars (Hardy *et al.*, 1996) and lower branches that dip into the ponds (O'Gara, 1998) that become infected. The new inoculation technique (Lucas *et al*., 2002) simulates the ponding of stems prior to infection but dispenses with the need for water receptacles and production of zoospores (O'Gara *et al*., 1996), making it an effective but quicker alternative for inoculation without wounding. The technique could be considered in studies requiring stem inoculation with *Phytophthora* spp. and other pathogens in agricultural and horticultural situations and in natural ecosystems.

11.3 Water status and timing of inoculation with *P. cinnamomi*

Timing of inoculation in relation to the watering regime had a major effect on the percentage of plants infected, regardless of genotype. Two different timings of inoculation of droughted plants were employed in addition to the inoculation of plants kept at container capacity without droughting (Fig. 11.1). Most recoveries of *P. cinnamomi* were made and most deaths and colonization were observed in plants which had been underbark-inoculated 7 days prior to droughting (Chapter 3). Because of the high proportion of deaths in that experiment, the new inoculation technique was developed (Chapter 4), and the immediate implementation of droughted conditions at the time of inoculation was trialled (Chapters 6 and 7). In Chapter 8, plants were inoculated after a period of drought, immediately after being restored to container capacity. In all experiments some plants had been kept at container capacity throughout the trial.

Regardless of genotype, fewer clonal plants became infected when inoculated immediately prior to droughting. This was a clear trend (Table 11.1). In contrast, more plants in all genotypes became infected if inoculated when restored to container capacity after a period of drought. This indicates firstly, that the plants show increased resistance when water stressed and secondly, that the pathogen, *in planta*, can respond rapidly to the increase in water status of the host. However the highest proportion of infection was in inoculated plants, which were kept at container capacity throughout the trial, or at container capacity for 7 days after inoculation, prior to droughting. Seven days of high water status was ample time for the pathogen to colonize host tissue before the gradual decline to droughted conditions, especially in the underbark inoculated plants (Chapter 3). This supports the original hypothesis that droughted *E. marginata* plants will have more resistance to *P. cinnamomi* than plants at container capacity.

The timing of defence responses, the ability of the host to recognize the pathogen, the synthesis of phenolic compounds necessary for the deposition of suberin and lignin which provide physical barriers to the pathogen's progress, in addition to constitutive phenolic compounds, and gene expression are all factors in effective defence (Bowles, 1990). Many publications (reviewed by Boyer, 1995)

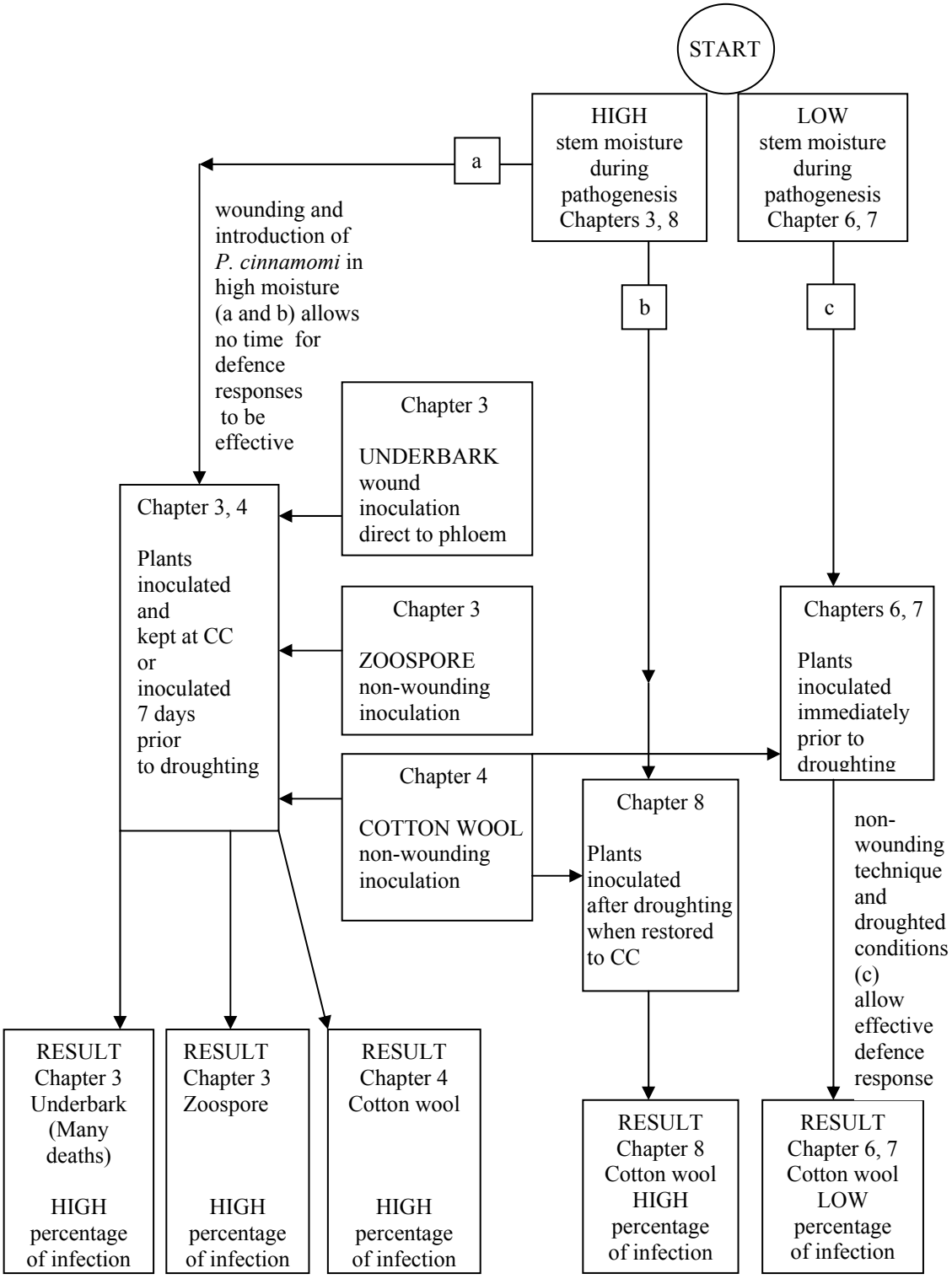


Figure 11.1 The importance of timing of inoculation. Overview of the influence of different inoculation techniques, applied at different times in relation to the implementation of different watering regimes on the subsequent disease development after inoculating *E. marginata* plants with *P. cinnamomi* in glasshouse trials. Of the 3 main pathways, a, b, and c which are indicated at the start, less infection resulted in c, plants inoculated, without wounding, prior to droughting. CC = Container capacity.

Table 11.1 Summary of all glasshouse experiments comparing the proportion of *E. marginata* plants infected with *P. cinnamomi* in each genotype and watering regime to the timing of inoculation.

Genotype *	Inoc. method **	Watering regime ***	Timing of inoculation ****	Mean colonization (cm)	Infected plants %	Days to harvest	Chapter
S	UB	1	0	12.10	100	35	3
S	CW	1	0	4.67	94	14	4
S	UB	2	1	8.19	87	35	3
SS	CW	1	0	5.50	87	14	8
S	Z	2	1	6.64	86	35	3
RR	CW	1	0	3.25	75	14	8
S	Z	1	0	4.57	71	35	3
S	Z	2	1	6.06	67	63	3
S	CW	1	0	1.75	62	14	8
RR	CW	1	0	1.20	60	86	6
SS	CW	1	0	4.65	60	63	7
S	Z	1	0	8.33	56	63	3
S	Z	3	1	5.39	56	63	3
S	CW	3	3	1.20	50	14	8
SS	CW	3	3	2.80	50	14	8
RR	CW	3	3	2.29	50	14	8
RR	CW	2	2	1.43	43	66	6
RR	CW	1	0	1.40	40	66	6
SS	CW	3	2	0.40	30	63	7
RR	CW	2	2	0.80	20	86	6
RR	CW	3	2	0.40	20	86	6
SS	CW	2	2	0.60	20	63	7
RR	CW	3	2	0.20	20	66	6
RR	CW	1	0	0.10	10	108	6
RR	CW	3	2	0.30	10	108	6
RR	CW	2	2	0.00	0	108	6

* SS = *E. marginata* plants susceptible to *P. cinnamomi*,
RR = *E. marginata* plants resistant to *P. cinnamomi*, S = *E. marginata* seedlings.

** Inoc = Inoculation, UB = underbark inoculation,
CW = cotton wool inoculation, Z = zoospore inoculation.

*** (1) plants were kept at container capacity throughout the trial (2) plants were droughted to wilting point and maintained at a droughted level until harvest and (3) plants were droughted as in (2) but restored to container capacity after a period of drought.

**** (0) plants were kept at container capacity and the timing of inoculation was not applicable (1) plants were inoculated 7 days prior to droughting (2) plants were inoculated immediately prior to droughting and (3) plants were inoculated immediately after restoration to container capacity after a period of drought.

report that increased physiological stress in a droughted plant weakens the host, predisposing it to attack by pathogens, which may be already present. However, because *P. cinnamomi* needs water for survival, mycelial growth and for reproduction (Zentmyer, 1980), the lower water status resulting from drought appears to disadvantage *P. cinnamomi* growth *in planta.*

The length of time from inoculation to harvest is also an important consideration. Several researchers have found that recovery of *P. cinnamomi* from infected plants (Davison *et al*., 1994; O'Gara, 1998; McDougall *et al.,* 2002) and soil (Duncan and Keane, 1996; Weste and Kennedy, 1997) decreases with time. Greater proportions of recoveries in RR and SS clonal plants were from plants harvested 14 days after inoculation and the least recovery of *P. cinnamomi* was from plants resistant to *P. cinnamomi* and harvested at the longest time (108 days) after inoculation. The possible reasons for this decrease are discussed in the next section (11.4).

In some glasshouse studies (Chapters 6 and 7), plants were inoculated prior to imposition of water deficit. This was modelled on seasonal increases of inoculum in the field during winter and spring rains (Shearer and Shea, 1987) which are normally followed by up to five months of summer drought. However, infection in the field can occur after droughting, when the occurrence of a summer rainfall event (Chapter 5) or irrigation (Shearer and Shea, 1987) provides temperature and moisture conditions conducive to the germination of infective propagules of *P. cinnamomi* in the soil, e.g. after high summer rainfall in 1982 (Tippett and Hill, 1983; Tippett *et al*., 1985). The abnormally heavy summer rainfall, after inoculation of plants, invalidated the droughted treatments in the field trial (Chapter 5), but presented an unexpected opportunity to observe the development of disease in these conditions, which are unusual for a region with a mediterranean climate and long hot dry summers. The sudden increase in disease development in the February harvest after the January rainfall was symptomatic of *P. cinnamomi* breakout from containment by the host, after high summer rainfall (Tippett *et al*., 1985). This highlights the importance of land preparation to avoid ponding in riplines in rehabilitated mine sites. The observations in the field after the summer rainfall event (Chapter 5) led to further studies of the interaction of *P. cinnamomi* and droughted plants in the

glasshouse (Chapters 6, 7 and 8) and to a series of trials to improve methods of recovery of *P. cinnamomi* (Chapter 9).

11.4 Recovery of *P. cinnamomi*

The disease was not always expressed with surface lesions, therefore colonization of plant tissue was the main determinant in assessing the extent of infection. Agar, selective for *Phytophthora*, facilitates the isolation and recovery of the pathogen (Tsao and Guy, 1977; Hüberli *et al*., 2000). However, when conventional methods of isolation failed to recover the pathogen from lesioned and obviously infected tissue, falsely negative observations were suspected. This issue was raised by Hüberli *et al*. (2000) and further addressed in the current study with an investigation of inhibitory factors (Chapter 9). The most effective method which facilitated recovery of the pathogen was to leach inhibitory compounds from the plant tissue by a series of immersions in water prior to plating onto selective agar. This had been established by O'Gara (1998) and Hüberli *et al*. (2000) and is supported by the current study. In addition, this study clearly demonstrated the inhibitory effect that diluted exudates from infected tissue had on the growth of *P. cinnamomi in vitro* and investigated the use of additives to the media to reduce the inhibition. Further research to determine methods to counter the effect of undiluted exudates from infected tissue will increase the accuracy of recovery data. This has important implications for decisions in mining, forestry, agricultural or environmental management reliant on the accurate assessment of tracts of land where the presence of *P. cinnamomi* is a potential threat. This is especially true in Western Australia where infection in spring, followed by summer drought, can lead to containment of the pathogen by the host. The host may exhibit no obvious symptoms of disease until conditions become conducive at a later date. This was demonstrated when the appearance of surface lesions and an increased recovery of *P. cinnamomi* from resistant clonal *E. marginata* plants was recorded in the field in the February harvest after high summer rainfall (Chapter 5).

Lack of recovery does not necessarily prove lack of infection. Immersion in water leaches the inhibitory compounds from the woody stem tissue, but if the stem segments are thick, the pathogen will have enough nutrients available within the host

tissue. It can adapt from a biotrophic (*in vivo*) to a saprophtytic (after harvest) phase and outgrowth onto nutrient agar may not result during the time that agar plates are monitored. The pathogen has the ability to survive saprophytically (Zentmyer and Mircetich, 1966; Reeves, 1975; Weste, 1983). *P. cinnamomi* has been recovered from dead *E. marginata* trees, where deaths were not recent (Blowes *et al*., 1982; Hardy *et al*., 1996). Outgrowth is more likely from thin, small sections of infected stems where the pathogen has immediate access to an alternative nutrient supply in the agar. It could also be hypothesized that the inhibitory compounds in infected stems (Chapter 9), can (a) induce dormancy in the pathogen or (b) have a fungistatic effect. If either of these outcomes is the reason for the lack of growth onto agar and subsequent mis-diagnosis of the absence of *P. cinnamomi*, then an effective procedure to break the dormancy needs to be developed. The leaching of inhibitory compounds only partly addresses this problem (Chapters 5, 6, 8 and 9 in this thesis; also O'Gara, 1998; Hüberli *et al*., 2000).

11.5 *E. marginata* and resistance to *P. cinnamomi*

Resistance and susceptibility to disease varies with genotype within this species (McComb *et al*, 1990; Cahill *et al*., 1993; Stukely and Crane 1994) but infection and disease development does not inevitably result in the death of the host. Given time to synthesize lignified tissue, host plants can contain the pathogen. *P. cinnamomi* has infected resistant clonal plants in the field, with the death of some individuals (Chapter 5). Disease development was facilitated by the summer rainfall event, but recovery of the pathogen diminished over time, indicating containment by the resistant host plants. In contrast, in the same period of time (from February to August) and in a nearby location, disease was expressed in increasing proportions in an area planted with *E. marginata* seedlings. These very different outcomes attest to the value of the research that produced clonal plants resistant to *P. cinnamomi* (McComb *et al*., 1990; Cahill *et al*., 1992; Stukely and Crane, 1994) and to the contribution of these plants in mine site rehabilitation. However, a different isolate of *P. cinnamomi* from that used to inoculate the resistant clonal plants was responsible for the deaths of the seedlings and isolates have been shown to vary in pathogenicity (Dudzinski *et al*., 1993; Hüberli 1995; Hüberli *et al*., 2001).

The ability of additional levels of a phenolic compound, catechol, to inhibit the colonization of *E. marginata* roots by *P. cinnamomi* was investigated (Chapter 10). It was hypothesized that, if roots could uptake the catechol, increased levels of phenolic compounds *in planta* might provide increased resistance to the pathogen. Increasing levels of catechol were shown to reduce the extent of colonization by *P. cinnamomi*. The results encourage further research because there is no research on the uptake of phenolic compounds by the roots of *E. marginata* and little comparable research with other species (Retig and Chet, 1974; Carrasco *et al*., 1978).

11.6 Other factors affecting the development of disease

Interactions of the pathogen with the moisture content, the biochemical and physical defenses of the host in addition to the prevailing edaphic and climatic conditions form a complex array of dynamics with numerous possible outcomes. In addition, the virulence and pathogenicity of different isolates of *P. cinnamomi* can vary (Hüberli, 1995). Ambient temperature is also a determining factor in *P. cinnamomi* growth (Zentmyer, 1980, Hüberli, 1997; R. Pilbeam *pers. comm*.). Temperatures in the field (Chapter 5) were highest in December. In the exposed rehabilitated mine site pit, surface soil temperature and ambient temperature are higher than forest conditions (McChesney, 1995). This may explain the low recovery of the pathogen in December. Stoneman (1992) found that soil moisture in open sites was greater with less evapotranspiration from tall canopy species. In forested areas, *E. marginata* trees in low-lying sites have been reported to be more prone to infection by *P. cinnamomi* than upland sites (Shearer and Shea, 1987). Deaths of trees may also be attributed to waterlogging or to a combination of waterlogging and *P. cinnamomi* infection (Davison, 1997). Ponded riplines in the mine site initially suggested waterlogging. The possibility of waterlogging in low lying areas and the associated oxygen deficiency confounds the field observations, introducing another stress, and contributing additional input to the debate on the interaction of *P. cinnamomi* and *E. marginata* and the development of disease and death in susceptible plants (Davison, 1997). However, it has been shown that ponding can be contained within the ripline and that the soil below the ponded area is not necessarily waterlogged (Burgess *et al*., 1999b).

11.7 Drought and *P. cinnamomi*

Drought and the adaptation of plants in the south-west of Western Australia to drought may be factors that hinder the ability of *P. cinnamomi* to kill some infected hosts, such as *E. marginata*, which are endemic to this locality. These results help to explain why outbreaks of *P. cinnamomi* are more severe in years when there is summer rainfall. Not only is the pathogen more abundant, but the plant's level of resistance is lower than in dry years. Symptoms of disease in *E. marginata* can take a few years to manifest and mass collapses of *E. marginata* were seen in the early 1960s and in 1982-1984 (Shearer and Tippett, 1989) following heavy summer rainfall in 1955 and in 1982 (Davison, 1997). Increased disease development was also seen in the field trial following the heavy summer rainfall of January 2000 (Chapter 5). The ability of *E. marginata* to transpire throughout summer (Doley, 1967; Colquhoun *et al*., 1984) has been documented and osmotic adjustment by this species in droughted conditions (Stoneman, 1992) was demonstrated again in Chapter 2. The summer rainfall event negated the comparison between the irrigated plants and the plants intended to experience drought in the field trial, but simulated drought in the glasshouse experiments showed that droughted plants were less colonized than the plants which were kept at container capacity. In European studies, less development of disease caused by *P. cinnamomi* was seen in droughted *Quercus* spp. (oak) than in watered plants of the same species (Robin *et al*., 2001). Luque *et al*. (1999) describe the negative effects of *P. cinnamomi* on photosynthesis and water transport in *Quercus suber* and cite Brasier (1993) and Wargo (1996) who suggest that *P. cinnamomi* may predispose plants to drought. Lesion growth was less in water stressed *Eucalyptus sieberi* seedlings infected with *P. cinnamomi* than in frequently watered seedlings (Smith and Marks, 1986). However, Dawson and Weste (1982) report on the difference between infected *E. sieberi* (reduced water transport) and infected *E. maculata* (water transport unaffected). In infected *E. marginata* Cahill *et al*. (1986 b) found reduced cytokinin production in the xylem tissue but not in infected roots of the more resistant *Corymbia calophylla*, and extrapolated these finding to suggest that water transport in *E. marginata* was more likely to be affected after infection by *P. cinnamomi*. The increased presence of

tyloses in the xylem of infected *E. marginata*, compared to control plants, can also restrict water transport (Davison, 1994). These observations support the argument that *P. cinnamomi* can predispose some plants to damage caused by drought, rather than drought predisposing plants to *P. cinnamomi*. They also illustrate the fact than conclusions drawn from comparisons between species, even within the same genus, may be untenable.

It would be of interest to repeat the field trial (Chapter 5), in a year when summer rainfall did not occur, to test the original hypothesis that, in field conditions, disease development in droughted *E. marginata* plants is not as extensive as in irrigated, non-stressed plants. Alternatively, if summer rainfall was high, plants could be inoculated again after the event or, after a simulated summer rainfall event with increased irrigation in both irrigated and droughted plots. Lignotuber susceptibility in *E. marginata* seedlings, in both droughted and irrigated conditions could also be investigated. Leaf axils were particularly vulnerable to *P. cinnamomi* infection (O'Gara 1998), so newly formed shoots growing from the lignotuber would also be prone to infection when *P. cinnamomi* is present in ponded riplines. The use of clonal plants of genotypes which are resistant to *P. cinnamomi*, though more costly, appears to be warranted. However, when planting or sowing to revegetate the mine site, increasing the density of plants in areas known to be infested with *P. cinnamomi* may also have greater long-term benefits. High plant density would increase soil water uptake and lower moisture content in the soil and *in planta*, would possibly decrease the spread of the pathogen. Some plants would not survive, but those that did would have not only varying degrees of resistance to *P. cinnamomi* but also a greater genetic diversity with attributes needed to withstand other challenges. Once established, the healthy plants could be selectively thinned. Remaining plants would experience reduced competition and would form the basis of a ecological community, resistant to *P. cinnamomi* and to drought.

11.8 Directions for future research

The current study has indicated directions for future research which include:

- Investigating the epidemiology of *P. cinnamomi* in mine site conditions

- Repeating of the field trial (Chapter 5) with resistant clonal *E. marginata* plants to test the original hypothesis that droughted plants will be less colonized than irrigated plants in rehabilitated mine site conditions in a normal season of summer drought
- Simulating summer rainfall and outbreak of *P. cinnamomi* with additional irrigation and re-inoculation of resistant *E. marginata* plants and *E. marginata* seedlings in a rehabilitated mine site to confirm theory of containment in resistant plants
- Investigating the susceptibility of the *E. marginata* lignotuber to *P. cinnamomi* in droughted and irrigated conditions in a rehabilitated mine site
- Using the new inoculation technique on susceptible hosts (e.g. *Banksia* spp. and other jarrah forest understorey species) in glasshouse trials and in mine site conditions to determine if susceptible hosts can survive when droughted immediately after becoming infected
- Comparing the stem moisture content of (1) *E. marginata* plants resistant to *P. cinnamomi* (2) *E. marginata* plants susceptible to *P. cinnamomi* (3) seedlings of *E. marginata* and (4) understorey species and correlating the results with disease development in all plants
- Testing of additives (e.g. other antioxidants) to the growth media to counter the inhibitory effects of exudates from infected stems of *E. marginata* to the *in vitro* growth of *P. cinnamomi*
- Determining the levels of phenolics in roots of *E. marginata* plants, pre-treated with additional catechol or another phenolic compound, to assess (1) the level of constitutive phenolics (2) the endogenous level of phenolics after uptake from solution (3) the level of phenolics synthesized *de novo* after inoculation with *P. cinnamomi* and (4) the degree of adherence of catechol to the root surface
- Determining, with scanning electron microscope studies, how *P. cinnamomi* hyphae invade the periderm when the new inoculation technique is used and
- Developing histological methods using vital stains to monitor necrosis of host cells as disease develops

11.9 Conclusion

The studies in this thesis have contributed to the understanding of the importance of water status to pathogenesis and disease development of *P. cinnamomi* in *E. marginata*, both in susceptible and in resistant plants. The hydrology of rehabilitated mine sites contributes significantly to the successful revegetation of mined areas. Reduction of ponded water in riplines and low-lying areas is essential to minimize disease. As global climate changes, the challenge for management of rehabilitated mine sites, forests and agricultural crops which are susceptible to *P. cinnamomi* will be to devise strategies to contain its spread and to minimize its impact by countering the multi-faceted survival strategies of this pathogen.

APPENDIX 1

Peat – Perlite Potting Mix

Component	Quantity	Product and/or Manufacturer
Peat	30 L	Floratorf
Perlite	20 L	Laporte Group Australia Willetton, W. Australia 6155
IBDU (Isobutylide diurea)	42.48 g	Mitsubishi Chemical Co. Tokyo, Japan
KNO3 Potassium nitrate	21.94 g	
Acid calcium phosphate	19.61 g	Aerophos. Wright and Wilson, Aust. Ltd Melbourne, Australia 3000
FeSO4 Ferrous sulphate	14.37 g	
FeO Ferric oxide	29.41 g	
Dolomite	39.22 g	
Gypsum	26.14 g	
Trace elements*	5.22 g	
Water	4640 ml	

* S 15%, Fe 12%, Ca 7.5%, Mg 5%, Mn 2.5%, Zn 1%, Cu 0.5%, Mo 0.5%, B 0.1%

Peat, perlite and nutrients were mixed thoroughly and water added.
When uniformly moist, the mix was transferred to clean hessian bags, then steam sterilized for 2 hours. It was used as a substrate for plants in glasshouse experiments.

APPENDIX 2

(a) V8 Nutrient Agar

Modified from Byrt and Grant (1979) and Hardham *et al*. (1991).

Component	Quantity	Manufacturer
V8 Juice (cleared*)	100 ml	Campbell's Soups Australia, Lemnos Road, Lemnos Vic. 3631, Australia.
CaCO3	0.1 g	
Bacto agar	17 g	Difco Laboratories Inc., Detroit MI 48232, USA
Sterile distilled water	900 ml	
β-sitosterol	0.02 g	Sigma Chemical Company, Sigma-Aldrich Pty Ltd., Castle Hill NSW 2154, Australia.

*The V8 juice was cleared by centrifuging at 5000 rpm for 20 minutes.
The supernatant was decanted and the pellet discarded. The supernatant was filtered through Whatman's No. 1 filter paper, using a Buchner funnel with vacuum pump attached. Aliquots of the filtrate (100ml) were transferred to plastic bottles and frozen at –20^{o}C for future use.

In the preparation of the agar, $CaCO_3$ was dissolved in 20 ml cleared V8 juice before it was added to the remaining V8 juice and water. The pH was adjusted to 6.0 and 10ml removed to a McCartney bottle before the agar was added. The agar solution and the V8 solution in the McCartney bottle were autoclaved at 121^{o}C for 20 minutes. β-sitosterol was dissolved in the cooled V8 solution to which a drop of Tween 80 detergent had been added. Contents of the McCartney bottle were added to the warm (~50^{o}C) V8 agar solution prior to it being poured into Petri dishes.

This agar was used for the growth of axenic cultures of *Phytophthora cinnamomi.*

(b) V8 Nutrient Broth

V8 Nutrient Broth, used in the preparation of zoospores, was prepared in the same manner with the exclusion of agar. Byrt and Grant (1979 found that the addition of β-sitosterol increased zoospore production.

APPENDIX 3

NARPH Agar, selective for *Phytophthora*

Shearer and Dillon (1995) Hüberli *et al*. (2000).

Component	Quantity	Manufacturer
Nystatin (Nilstat)	1 ml	Wyeth-Ayerst Australia Pty. Ltd. Baulkhan Hills, NSW. Australia.
Ampicillin sodium	100 mg	Fisons Pty. Ltd. Sydney, Australia.
Rifampicin (Rifadin)	0.5 ml	Hoechst Marion Roussel Australia Pty. Ltd. Lane Cove, NSW. Australia.
PCNB (Pentachloronitrobenzene) (Terraclor)	100 mg	Uniroyal Australia Pty. Ltd. Melbourne, Australia.
Hymexazol (Tachigaren)	50 mg	Sankyo Company. Tokyo, Japan.
Cornmeal agar (CMA) (Oxoid)	17 g	Unipath Ltd. Basingstoke, England.
Sterile distilled water	I L	

10 ml of the distilled water was transferred to a McCartney bottle.
The CMA was added to the remaining 900 ml of distilled water.
Both were autoclaved at 121^{o}C for 20 minutes.
The NARPH antibiotics were dissolved in the cooled 10 ml of sterile distilled water, added to the warm (~50^{o}C) agar solution and mixed well before the medium was poured into Petri dishes. This selective agar was used after re-passaging *P. cinnamomi* through a host (Appendix 9) and after harvesting plants which had been inoculated with or infected by *Phytophthora*.

APPENDIX 4

Karnovsky's fixative (modified)

Karnovsky (XXXX) and modified by Glauert (1975)

Component	Proportion
Formaldehyde	[2%]
Gluteraldehyde	[2.5%]
Phosphate buffer 0.1M	

APPENDIX 5

Production of *Phytophthora cinnamomi* zoospores

Method modified from Dolan and Coffey (1986) and O'Gara *et al*. (1996).

An isolate of *P. cinnamomi* was repassaged through host tissue.
After harvesting, segments of infected stem were plated onto NARPH selective agar.
An axenic sub-culture of *P. cinnamomi* was plated onto V8 nutrient agar (Appendix 2a).
The plate was sealed with Parafilm™ and placed in an incubator at 24±1°C where the isolate grew in the dark for 5 days.
In aseptic conditions, plugs of mycelial agar were cut from the prepared plates, placed in Petri dishes and covered with sterile nutrient V8 broth (Appendix 2b).
These were returned to the incubator for another 5 days.

Soil filtrate was then prepared. 100 g of soil, passed through a 10 mm sieve, was added to 1L of sterile distilled water (SDW), agitated, and left to stand overnight. It was then filtered through Whatman's No. 1 filter paper, in a Buchner funnel in a side-arm flask with a vacuum pump attached.

The V8 broth was removed from the mycelial plugs which became mats of mycelium with a profusion of hyphae. The mats were rinsed three times with SDW to remove all nutrients, then covered with 10 ml of the non-sterile soil filtrate. They were left in the soil filtrate, under lights, overnight (or longer), and, with a microscope, regularly checked for sporangia. When sporangia were observed, the mats were rinsed with SDW and cold shocked at 4°C for 30 minutes. The mats were then returned to the incubator at 24±1°C until zoospore release, about 30 minutes to an hour later (Fig. A5.1).
Zoospores can be motile for an hour or more before encysting.
The use of acid washed beakers and pipette tips will lessen the likelihood of encystment when zoospores come into contact with the surfaces of equipment. Concentration of zoospores in solution can be assessed by dropping a 5µl aliquot of solution onto a glass slide with fixative and counting the number of zoospores, then extrapolating to the required unit of measurement.

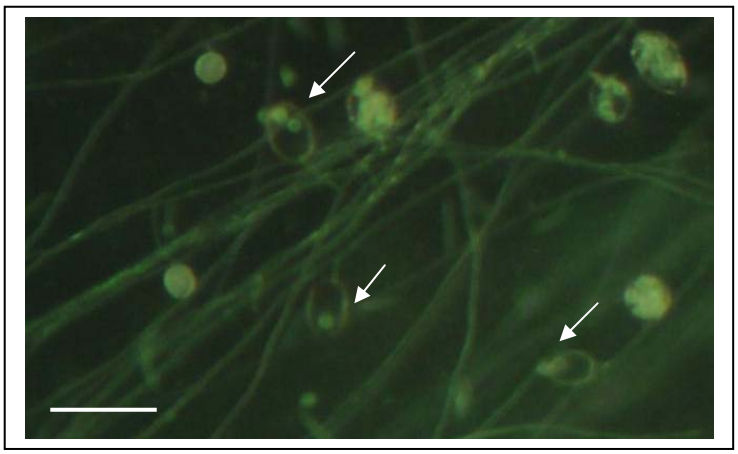


Figure A5.1 Mature sporangia of *Phytophthora cinnamomi* (arrowed) releasing zoospores into solution. Scale bar = 100 µm.

APPENDIX 6

Hydroponics Pilot Trial with Lupins

Acknowledgement

This work was part of a project undertaken by Emma Groves, a student at Murdoch University, and was funded with a Neville Stanley Studentship. The project was supervised by Dr Giles Hardy and Anne Lucas. It was used as a model for the experiments with jarrah seedlings (Chapter 10). Relevant details have been rewritten or edited by Anne Lucas, using the results obtained in Emma's work.

A6.1 Introduction

The phenolic compounds synthesised by plants are secondary metabolites which play a role in plant defence against herbivory or pathogenic attack. They can create physical or chemical barriers (Candela *et al*., 1995) and can be fungicidal or fungitoxic (Vidhyasekaran, 1997). Individual phenolic compounds, found in *E. marginata*, have been shown, *in vitro*, to have an inhibitory effect on the growth of *P. cinnamomi* (Cahill and McComb, 1992). Lupins, susceptible to disease caused by *P. cinnamomi*, were chosen for this pilot trial because the seeds germinate readily and the seedlings develop more rapidly than *E. marginata.* The experimental design and the hydroponics equipment were modified slightly for the work described in Chapter 10.

The aim of this pilot trial was to determine, with phenolic assays, if additional phenolic compounds could be taken up by the roots of seedlings. If they could, would the higher levels of phenolics *in planta* provide greater host resistance to disease when inoculated with the pathogen, *P. cinnamomi*?

A6.2 Methods

A6.2.1 *Experimental design*

Seedling roots were suspended in one of four concentrations (0, 10, 25 and 40 mg L^{-1}) of catechol (Sigma Chemical Company, Sigma-Aldrich Pty. Ltd., Castle Hill NSW, Australia 2154), and 5 replicates in each treatment were removed in each of 3 harvests. Harvest 1 was prior to the addition of catechol to the nutrient solution, Harvest 2 was after 48 hours of roots' immersion in catechol solution. At this point, seedlings were inoculated with *P. cinnamomi* and Harvest 3 was 4 days after inoculation. Controls were applied to all treatments.

A6.2.2 *Biological material*

Surfaces of lupin (*Lupinus augustifolius* var. *merrait*) seeds were sterilized by agitating in a 4% sodium hypochlorite (NaOCl) solution for 1 minute. They were rinsed

5 times in sterile distilled water (SDW), then, in aseptic conditions, placed on moist filter paper in 90 mm Petri dishes to be germinated in the dark at room temperature for 2 days. Germinated seeds were transferred to sterile moist filter paper in 150mm Petri dishes. Plates were placed at a 75^{o} angle, under a growth lamp in a controlled temperature room (24^{o}C), with a light/dark regime of 16/8 hours. Two days later, the rapidly growing seedlings were transferred to the hydroponics system.

A6.2.3 *Hydroponics system*

Ag-row nutrient solution (Aquaponics WA, Canningvale, WA 6155) was used to grow the seedlings in 26 x 2L plastic containers, which had been UV sterilized, and positioned under growth lamps (Fig. A6.1) in a controlled temperature room (24^{o}C). The nutrient solution was replaced each week and aeration was effected with a 0.5 cm diameter hose connected to a continuous supply of air. A purpose-made piece of polystyrene was floated on the surface of the solution. With the support of a sterile piece of cotton wool fitted around the stem, seedlings were inserted into holes in the polystyrene and roots were suspended in the nutrient solution. After Harvest 1, the nutrient solution was modified with the 4 different concentrations of catechol for 48 hours.

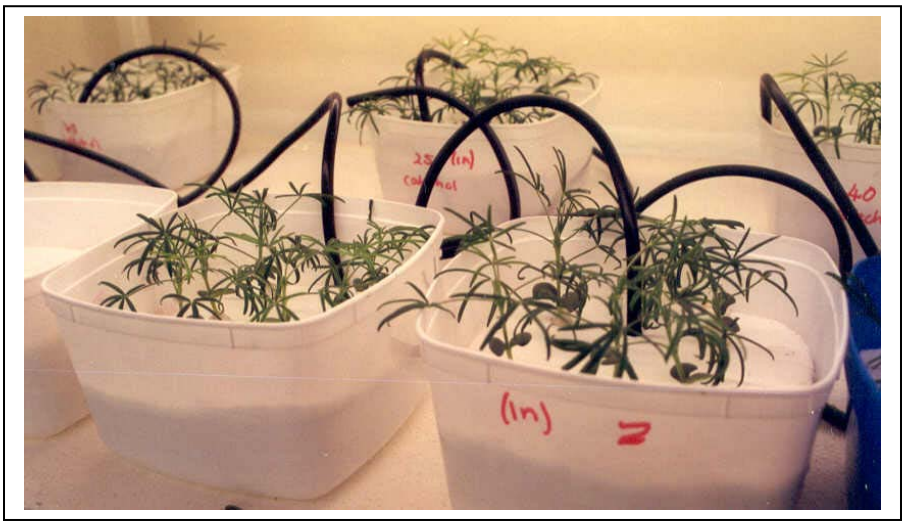


Figure A6.1 Lupin (*Lupinus augustifolius* var. *merrait*) seedlings growing in the hydroponics system.

A6.2.4 *Zoospore inoculation*

Zoospores of *P. cinnamomi* isolate MU 94-48 were produced using a modified method of O'Gara *et al*. (1996), (Appendix 5). Seedlings were removed from the hydroponics containers and placed on a tray lined with Gladwrap (Glad Products of Australia, Padstow NSW 2211). Root tips were inoculated with a 5µl suspension of zoospores and left to stand for 60 minutes before being returned to the hydroponics

system. Roots were covered with an extra film of Gladwrap and the leaves sprayed with SDW to avoid desiccation of leaves.

A6.2.5 *Phenolics assay*

Root material was cut into 1 cm segments from 4 cm above, and down to, the tip. The segments were powdered in liquid N with a mortar and pestle and 50mM phosphate buffer (pH 7) added. The extract was centrifuged at 4^{o}C for 10 minutes at 14,000g. This supernatant was used to determine the level of soluble phenolics. After the remaining pellet was washed with 5mM phosphate buffer (pH 7), surplus liquid was removed under vacuum and the pellet dried at 60^{o}C. It was weighed and 4M NaOH added. The pellet was boiled for 2 hours then centrifuged at 14,000g for 5 minutes. The resulting supernatant was used to determine the level of bound phenolics. Folin and Ciocalteu's Phenol reagent (Sigma Chemical Company, Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia 2154) was used to determine the phenolic content which was expressed as μmol / mg of dry weight by comparing the absorbance at 725 nm with that of a *p*-coumerate standard curve.

A6.2.6 *Statistical analysis*

The data were analysed with Microsoft Excel for Windows (Microsoft Corporation, USA).

A6.3 Results

A6.3.1 *Uptake of catechol by roots*

Levels of soluble phenolics increased in roots treated with 40 mg/L catechol. The level of soluble phenolics in catechol-treated roots varied with distance from the root tip. Highest levels were found at the tip. The level of phenolics in non-treated roots was relatively constant. There was no significant change in soluble phenolics in roots treated in catechol concentrations of 10 mg/L or 25 mg/L. The bound phenolics assay was abandoned when precipitation made readings inaccurate. The level of total phenolics for roots in the pilot trial was therefore unable to be obtained.

A6.3.2 *Extent of colonization by* **P. cinnamomi** *in roots*

The percentage of recovery of *P. cinnamomi* was less from roots treated with 40 mg/L catechol than from control plants, but there was no significant ($P>0.05$) reduction in colonization of *P. cinnamomi* in roots treated with catechol (Fig. A6.2).

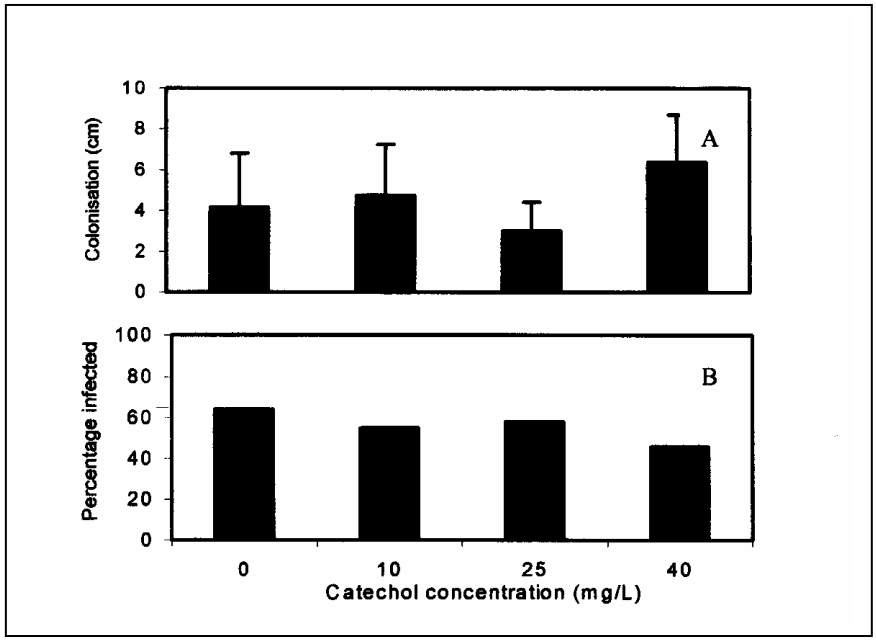


Figure A6.2 Effect of catechol on (A) colonization of lupin roots by *P. cinnamomi* and (B) the percentage of inoculated roots that became infected. Bars represent the standard error of the mean.

A6.4 Outcome of pilot trial

Fewer recoveries were made from roots treated with the highest concentration of catechol. Catechol treatments did not reduce levels of colonization by *P. cinnamomi* in the roots, though Retig and Chet (1974) found that tomato plants treated with higher levels of catechol were more resistant to disease caused by *Fusarium*. Only roots in the highest concentration had increased levels of soluble phenolics after inoculation. Whether this is the result of uptake of catechol by the roots, adherence of catechol to the epidermal surface, or the result of *de novo* synthesis of phenolic compounds, as a defence strategy by the plant, is undetermined.

The pilot trial indicates that little increase in resistance to disease caused by *P. cinnamomi* can be expected with low concentrations of catechol. Trials with jarrah seedlings were designed with higher concentrations. The hydroponics equipment and the methodology employed in the pilot trial worked well and, with minor modifications, was used in the study with jarrah seedlings (Chapter 10).

APPENDIX 7

Effect of an antioxidant on the *in vitro* growth of *P. cinnamomi*. A pilot trial.

A7.1 Introduction

The aim of this pilot trial was to monitor the *in vitro* growth of *P. cinnamomi* on NARPH agar amended with different concentrations of ascorbic acid, prior to conducting the experiment described in Chapter 9c.

A7.2 Method

A7.2.1 *Experimental design and materials*

NARPH selective agar (Appendix 3) was amended with six different concentrations (0 mg L^{-1},100 mg L^{-1}, 500 mg L^{-1}, 1000 mg L^{-1}, 1500 mg L^{-1} and 2000 mg L^{-1}) of ascorbic acid (Sigma Chemical Co., Sigma-Aldrich Pty.Ltd., Castle Hill, NSW, Australia. 2154). A sub-culture of *P. cinnamomi* isolate 94-48 was prepared and a colonized plug 5 mm in diameter was placed in the centre of Petri dishes containing 20 ml of amended agar. There were 6 replicates of each concentration.

A7.2.2 *Preparation*

Preparation of media, culture of *P. cinnamomi,* assessment of pH of media and statistical analysis for the pilot trial were as described in Chapter 9c.2 (Methods).

A7.3 Results

There was a significant (*df* 5, 30 ; $P<0.001$) difference in mycelial growth of *P. cinnamomi* between the concentrations of ascorbic acid (Tables A7.1 and A7.2) and a strong correlation ($r = 0.94$) between the mean diameter of growth and the pH in each concentration.

Table A7.1 Mean diameter of mycelial growth of *P. cinnamomi* on NARPH agar amended with different concentrations of ascorbic acid.

Basic medium	Concentration of ascorbic acid mg L^{-1}	Replicates n =	Mean diameter of mycelial growth (mm)	pH of agar
NARPH	0	6	76.67	5.3
NARPH	100	6	69.42	5.0
NARPH	500	6	63.75	3.9
NARPH	1000	6	36.75	3.1
NARPH	1500	6	23.42	2.9
NARPH	2000	6	18.83	2.9

A7.4 Outcome of pilot trial

This pilot trial was repeated in the main experiment (Chapter 9c) on agar amended with the same series of concentrations of ascorbic acid. To further investigate the effect that the pH of the media had on the mycelial growth, the pH of half the replicates in the main experiment was standardized to 6.0 and that of the other half was not adjusted.

APPENDIX 8

Germination of *Eucalyptus marginata* seed.

A8.1 Introduction

Seedlings required for the hydroponics experiments described in Chapter 10 were grown from seed germinated *in vitro*. The successful germination of seeds is influenced by moisture, temperature and light, as well as dormancy and viability. Fire plays a major role in germination of seeds of many Australian species and *E. marginata* seeds have been found to have a significant increase in germination in response to smoke treatments (Roche *et al*., 1997). In laboratory testing, the age of the seed and storage conditions must also be considered. Previous studies which investigate the effects of these often interacting factors have been reviewed (Bell, 1999). Percentages of germination of *E. marginata* seeds were greater in full darkness than in a 12 hour diurnal light period (Bell, 1994) or in white light and other wavelengths of light (Rokich and Bell, 1995) and at temperatures of 13 to 16^{o}C (Bell, 1994). These conditions of full darkness and low temperatures were applied to the seeds in the current experiments.

The aims of this experiment were to establish the germination requirements and the viability of the *E. marginata* seed

A8.2 Methods

A8.2.1 *Experimental design*

After a selection process (seeds that floated or sank) during surface sterilization, batches of *E. marginata* seed were subjected to 2 different pre-treatments (soaking in aerated water for 18 hours or not soaking) before *in vitro* germination (Table A8.1). The percentage of germination of each batch was calculated after 14, 21 and 28 days (Fig. A8.1).

Table A8.1 Protocol for pre-treatment and *in vitro* germination of *E. marginata* seeds.

Tr	Seeds prepared (*n* =)	Surface sterilized	Sank or floated when rinsed	Soaked in aerated water	Germinated at 28 days (*n* =)	Germinated at 28 days %
A	35	+	sank	+	33	94.29
B	35	+	sank	-	27	77.14
C	90	+	floated	-	51	56.67
D	260	+	combined sank/ floated	+	162	62.31

Tr = Treatment. Seedlings produced by Treatments A, B and C were used in the experiment described in Chapter 10a and those in Treatment D were used in the experiment described in Chapter 10b.

A8a.2.2 *Pre-treatment and germination of seeds*

E. marginata seeds from a seed mix (provenance Nanga, Alcoa Zone 5) obtained from Alcoa World Alumina, Australia were surface sterilized by immersion in a 1% NaOCl solution with continual agitation for 5 minutes. Aseptically, excess solution was removed by placing seeds over sterile filter paper in a Buchner funnel on a side-arm flask to which a vacuum line was attached. To ensure all traces of NaOCl were removed, seeds were rinsed 3 times by immersing the filter paper with seeds in beakers of sterile distilled water (SDW) and returning between rinses to the vacuum. All distilled water, filter papers and equipment were autoclaved at 121^{o}C for 20 minutes on 3 consecutive days before use.

Seeds that floated on the surface of the NaOCl solution or in subsequent rinses were separated from those that sank. Half the seeds that sank were transferred to a 1L flask of SDW which was aerated for 18 hours to encourage imbibition by the embryos. Water was drained away and these seeds were placed on a double layer of filter paper, wet with SDW, in 90mm Petri dishes. The Petri dishes were sealed with Parafilm™ and wrapped in foil and left in the dark at 16±2^{o}C for seeds to germinate. The other half of the seeds that sank and all the seeds that floated were placed on wet filter paper and germinated in the same conditions as the seeds soaked in aerated water.

The number of seeds germinated was noted at 14 days, 21 days and 28 days. Seeds were considered to have germinated when the radicle was visible. Cotyledons appeared and after 21 days true leaves were forming. When cotyledons appeared, seedlings were transferred to 150mm Petri dishes on 125mm filter paper (Whatman's No.1). Ten seedlings of similar size and age were positioned across the diameter of the base filter paper in the Petri dish. The developing root system was protected from light by folding another filter paper in half and covering the roots while the cotyledons emerged above. Sterile distilled water kept the filter papers wet and sealing the Petri dishes minimized loss of moisture. The Petri dishes were stacked at an 80^{o} angle under fluorescent light (40 μE m^{2} sec^{-1}) at 24^{o}C for 14 days before seedlings were transferred to the hydroponics system.

A8.3 Results

A8.3.1 *Seed germination and growing conditions*

The mean weight of all selected *E. marginata* seeds in Treatments A, B and C used in Experiment 10a (n = 160) was 10.34±0.14 mg and the mean weight of seeds in Treatment D used in Experiment 10b was 9.99±0.16 mg. The highest percentage of germination (94.29%), 28 days after preparation, was of seeds in Treatment A (n = 35) that sank into solution in the surface sterilization process and were then soaked in aerated water overnight before incubation. Seeds that sank but were not soaked before incubation, Treatment B, (n = 35) and seeds which did not sink, but floated on the surface of the solution, Treatment C, (n = 90) had germination, at 28 days, of 77.14% and 56.67%, respectively. There was deceptively little difference in the percentage of germination between seeds that sank and were soaked in aerated water (57.14%) and seeds that floated and were not soaked (52.22%) after 21 days, but a much greater difference was apparent after the final 7 days of monitoring (Fig. A8.1).

Seeds were prepared for Treatment D, used in Experiment 10b, at Day 21 of the first germination trial. (Treatments A, B and C). Percentage germination was calculated 28 days after pre-treatment, though in all treatments, seeds continued to germinate after

Day 28. After seeds germinated in the dark, seedlings developed healthy green cotyledons and true leaves when transferred to 150mm Petri dishes under fluorescent light at 24°C. They survived with roots in SDW without added nutrients for 14 days.

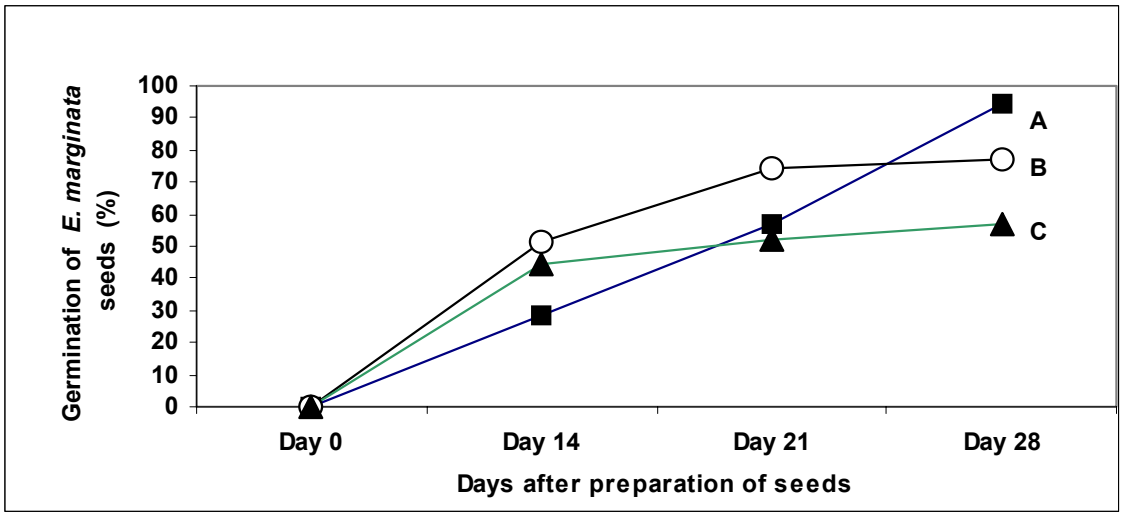


Figure A8.1 Germination of *E. marginata* seeds. Highest percentage of germination at Day 28 was in Treatment A.
A = seeds that sank during the preparation and were aerated for 18 hours (n = 35).
B = seeds that sank but were not aerated (n = 35) and
C = seeds that floated and were not aerated (n = 90).
Seeds were prepared for Treatment D at Day 21 of the first germination trial.

A8.4 Discussion

It was shown that the seeds were viable and that pre-treatment (soaking in aerated water after surface sterilization) improved the percentage of germination. The selection of seeds which sank in solution during surface sterilization may also contribute to a higher percentage of germination. The weight could indicate that these seeds can provide the embryo with a greater store of nutrients during the germination process. The percentage of seeds which germinated in Treatment A after being soaked in aerated water was greater than that of seeds which were not soaked. Surface sterilization with NaOCl may have made the testa more permeable, facilitating increased imbibition by seeds in the movement of the aerated water compared to imbibition on wet filter paper in still conditions. Seeds for Experiment 10b (Treatment D) were prepared at Day 21 of the first germination trial when the effect of separating sinking and floating seeds had not become apparent (Fig. A8.1). Seeds in Treatment D were surface sterilized and soaked in aerated water prior to incubation but the percentage of germination was much lower than that of seeds given the same pre-treatment inTreatment A. The inclusion of both floating and sinking seeds may have contributed to the lower percentage of germination in Treatment D. Conditions of full darkness and low temperatures proved conducive to the germination of *E. marginata* seeds, supporting the results reported by other researchers (Bell, 1994; Rokich and Bell, 1995). It has been suggested that the optimal temperatures for germination of *E. marginata* seed (13-16°C) are the same as winter temperatures of the jarrah forest in the mediterranean climate of the south-west of Western Australia, when rainfall is most likely to be highest (Bell, 1994).

APPENDIX 9

Assessment of the advantage of re-passaging an isolate of *Phytophthora cinnamomi*, prior to its use as inoculum.

A9.1 Introduction

Continued sub-culturing of *Phytophthora* can lessen its aggressiveness when introduced to a host plant (Jeffrey *et al*., 1962; Erwin, 1995).

The aim of this trial was to assess the effect that continued sub-culturing had on the growth of mycelia of *P. cinnamomi* isolate MU 94-48, the isolate used in all studies in this thesis.

A9.2 Methods

A9.2.1 *Experimental design*

Five replicates of 2 sub-cultures of *P. cinnamomi* isolate MU 94-48 were grown on V8 agar (Appendix 2a). PC 1 was the first sub-culture on V8 agar from the initial axenic culture obtained on NARPH selective agar (Appendix 3), after re-passaging the isolate through a host seedling (*Eucalyptus marginata*). PC2 had been sub-cultured several times on V8 agar over 6 months. The growth, on V8 agar, of mycelia of the isolate at 2 different levels of sub-culturing, was compared.

Table A9.1 Protocol for the growth of an isolate of *P. cinnamomi* (MU 94-48) at different levels of sub-culture.

Isolate	Time elapsed since re-passaging	Growth medium	Number of Replicates	Length of observation
MU 94-48 PC1	1 week	V8 agar	5	5 days
MU 94-48 PC2	6 months	V8 agar	5	5 days

A9.2.2 *Preparation of growth media*

Two media were prepared; NAPRH agar (Appendix 3), selective for *Phytophthora* (Hüberli *et al*., 2000) was used to prepare an axenic culture of *P. cinnamomi* isolate MU 94-48 after re-passaging (A9.2.) and V8 nutrient agar (Appendix 2a) was used to grow both sub-cultures of the isolate. Aseptically, syringes dispensed 10 ml of the agar into 90 mm Petri dishes. Swirling the liquid agar in the plates produced a thin, uniform film of agar. Plates were incubated at 24^{o}C for 2 days prior to the sub-culturing of the isolate to confirm the absence of contaminants.

A9.2.3 *Re-passaging of isolate through a host plant*

An *E. marginata* seedling was inoculated using the underbark inoculation technique, where an incision was made in the stem, extending into the phloem (Chapter 3). A plug of V8 agar, colonized by *P. cinnamomi* isolate MU 94-48, was inserted into this cut and covered with sterile wet cotton wool. Parafilm™ was wrapped around the stem, securing the agar and the cotton wool. It also sealed the wound area, limiting

desiccation of the colonized agar and any access to the wound by other pathogens.

Lesions were visible 4 days later and the plant was harvested. The stem was cut into 0.5 cm segments and plated onto NARPH selective agar. From the leading edge of the resulting outgrowth of mycelium, the *P. cinnamomi* was subcultured again onto NARPH and an axenic culture was confirmed by identification with a compound microscope (Olympus BH-2), magnification x200.

A9.2.4 *Growth of isolate on agar*

Aseptically, colonized agar plugs, 3 mm x 3 mm, were taken of the isolate and placed in the centre of 5 x 90 mm Petri dishes containing V8 agar. Five replicates of both levels of the sub-cultures were made (Table A9.1). Plates were sealed with Parafilm™ and incubated in the dark at 24±1^{o}C and monitored daily for 5 days. Two diameters of the mycelial growth, at right angles to each other, were recorded, and mean diameter calculated.

A9.3 Results

A9.3.1 *Growth of* P. cinnamomi *mycelia on V8 agar*

After 5 days the growth of *P. cinnamomi* mycelium was significantly (*df* 1,8; P<0.001) different between sub-cultures PC1 and PC2. PC1, the re-passaged sub-culture grew faster than PC2, which had been kept in storage and sub-cultured over 6 months (Fig. A9.1).

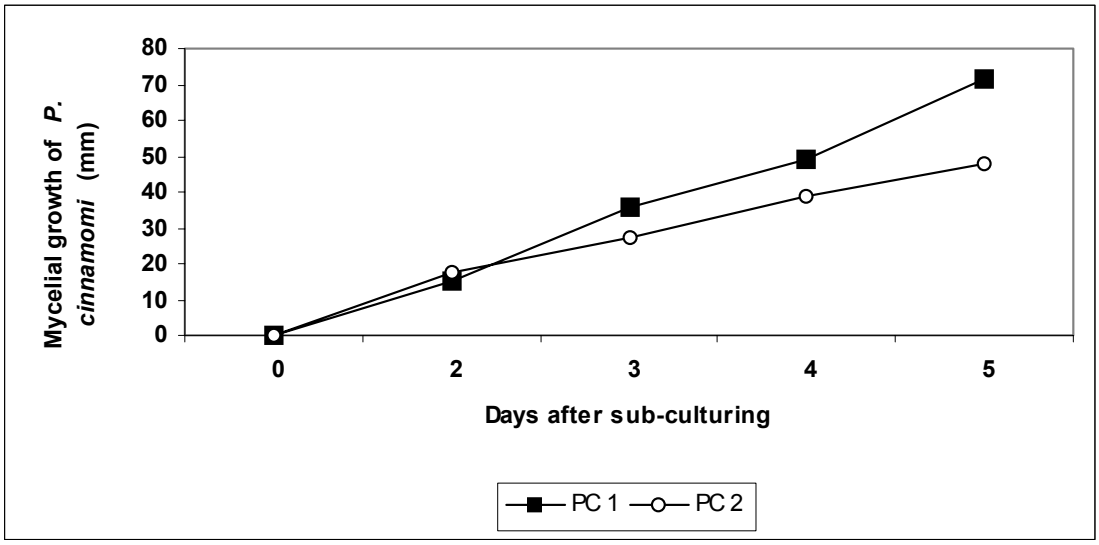


Figure A9.1 Growth of mycelia of two sub-cultures of *P. cinnamomi* on V8 agar. PC 1 was the first sub-culture after re-passaging through an *E. marginata* seedling and PC 2 had been sub-cultured many times and stored over a six-month period.

A9.4 Outcome

Mycelium of the re-passaged sub-culture of isolate MU 94-48 grew at a faster rate than the stored and often re-plated sub-culture. These *in vitro* results suggest re-passaging through a host plant reinvigorates the inoculum. Re-passaging of the isolate was a standard technique in the preparation of inoculum in all experiments described in this thesis.

References

Armstrong, P. (1979) - Biogeography. Themes and case studies. In 'Western Landscapes' (Ed. J. Gentilli) University of Western Australia Press, Nedlands, W.A. 6009.

Auckland, C. (2002) - The dilution of phosphite in rapidly growing plants and how soil and plant phosphate levels interact with phosphite and its ability to control *Phytophthora cinnamomi* Rands. Honours Thesis, Murdoch University, Perth, Western Australia.

Bartnicki-Garcia, S. and Wang, M.C. (1983) – Biochemical aspects of morphogenesis in *Phytophthora*. In: '*Phytophthora* – Its Biology, Taxonomy, Ecology and Pathology' Eds. D.C. Erwin. S Bartnicki-Garcia and P.H. Tsao. The American Phytopathological Society, St Paul, Minnesota USA. pp 121-137.

Bartle, J. and Slessar, G.C. (1989) – Mining and rehabilitation. In: 'The Jarrah Forest. A complex mediterranean ecosystem' (Eds. B. Dell, J.J. Havel and N. Malajczuk.) pp 41-51. Dordrecht: Kluwer Academic Publishers.

Batini, F. (1992) - Planning operational hygiene in CALM. In 'Dieback – What is the future?' (Eds. The Northern Sandplains Dieback Working Party) Department of Minerals and Energy, East Perth W.A. 6004.

Bell, D.T. and Heddle, E.M. (1989) – Floristic, morphologic and vegetational diversity. In: 'The Jarrah Forest. A complex mediterranean ecosystem' (Eds. B. Dell, J.J. Havel and N. Malajczuk.) pp 53-66. Dordrecht: Kluwer Academic Publishers.

Bell, D.T. (1994) – Interaction of Fire, Temperature and Light in the Germination Response of 16 Species from the *Eucalyptus marginata* Forest of South-western Western Australia. *Australian Journal of Botany* **42**: 501-509

Bell, D.T. (1999) – The Process of Germination in Australian Species (Turner Review No. 1). *Australian Journal of Botany* **47** (4): 475-517.

Bennett, R.N. and Wallsgrove, R.M. (1994) – Tansley Review No. 72 Secondary metabolites in plant defence mechanisms. *New Phytologist* **125**: 617-633.

Biggs, A.R. and Miles, N.W. (1988) – Association of suberin formation in uninoculated wounds with susceptibility to *Leucostoma cincta* and *L. persoonii* in various peach cultivars. *Phytopathology* **78**: 1070-1074.

Blaker, N.S. and MacDonald, J.D. (1981) – Predisposing effects of soil moisture extremes on the susceptibility of Rhododendron to *Phytophthora* root and crown rot. *Phytopathology* **71** (8): 831-834.

Blowes, W.M., Heather, W.A., Malajczuk, N. and Shea, S.R. (1982) – The distribution of *Phytophthora cinnamomi* Rands at two sites in southern Western Australia and at Durras in south-eastern New South Wales. *Australian Journal of Botany* **30**: 139-145.

Bostock, R.M. and Stermer, B.A. (1989) – Perspectives on wound healing in resistance to pathogens. *Annual Review of Phytopathology* **27**: 343-371.

Bowles, D.J. (1990) – Defense-related proteins in higher plants. *Annual Review of Biochemistry* **59**: 873-907.

Boyce, M.C. and Bennett, I.J. (1996) – Complimentary role of micellar electrokinetic capillary chromatography and high performance liquid chromatography in the separation of plant phenolics. *Analytical Letters* **29** (10): 1805-1815.

Boyer, J.S. (1995) – Biochemical and biophysical aspects of water deficits and the predisposition to disease. *Annual Review of Phytopathology* **33**: 251-274.

Brasier, C.M. (1983) – Problems and Prospects in *Phytophthora* Research. In 'Phytophthora – Its Biology, Taxonomy, Ecology and Pathology' Eds. D.C. Erwin. S Bartnicki-Garcia and P.H. Tsao. The American Phytopathological Society, St Paul, Minnesota USA. pp 351-364.

Brasier, C.M. (1993) – *Phytophthora cinnamomi* as a contributory factor in European oak decline. In: 'Recent Advances in Studies on Oak Decline.' (Eds. N. Luisi, P.Lerario and A. Vannini.) University of Bari, Italy. pp.49-57. (cited by Luque *et al*., 1999.)

Brooker, M. and Kleinig, D. (1990) – Field guide to eucalypts. South-western and southern Australia. Inkata Press Pty. Ltd: Melbourne, Australia.

Bunny, F.J. (1996) – The biology, ecology and taxonomy of *Phytophthora citricola* in native plant communities in Western Australia. PhD Thesis, Murdoch University, Perth, Western Australia.

Bunny, F.J., Crombie, D.S. and Williams, M.R. (1995) – Growth of lesions of *Phytophthora cinnamomi* in stems and roots of jarrah (*Eucalyptus marginata*) in relation to rainfall and stand density in mediterranean forest of Western Australia. *Canadian Journal of Forest Research* **25**: 961-969.

Burgess, T., McComb, J., Hardy, G.E. StJ. and Colquhoun, I.J. (1998) – Influence of low oxygen levels in aeroponics chambers on eucalypt roots infected with *Phytophthora cinnamomi*. *Plant Disease* **82**: 368-373.

Burgess, T., McComb, J.A., Colquhoun, I. and Hardy, G.E. St.J. (1999a) – Increased susceptibility of *Eucalyptus marginata* to stem infection by *Phytophthora cinnamomi* resulting from root hypoxia. *Plant Pathology* **48**: 797-806.

Burgess, T., Collins, S., Hardy, G.E.St.J., McComb, J. and Colquhoun, I. (1999b) – A Survey of the Frequency, Duration and Oxygen Content of Surface and Sub-Surface

Water during Early Vegetation Rehabilitation at the Huntly Mine Site. Environmental Research Bulletin No. 28, Alcoa of Australia Limited, Applecross, Western Australia.

Byrt, P. and Grant, B.R. (1979) – Some conditions governing zoospore production in axenic cultures of *Phytophthora cinnamomi* Rands. *Australian Journal of Botany* **27**: 103-115.

Cahill, D., Wookey, C., Weste, G. and Rouse, J. (1986a) – Changes in mineral content of *Eucalyptus marginata* and *E. calophylla* grown under controlled conditions and inoculated with *Phytophthora cinnamomi*. *J. Phytopathology* **116**: 18-29.

Cahill, D.M., Weste, G.M. and Grant, B.R. (1986b) – Changes in cytokinin concentrations in xylem extrudate following infection of *Eucalyptus marginata* Donn ex Sm with *Phytophthora cinnamomi* Rands. *Plant Physiol*. **81**: 1103-1109.

Cahill, D., Legge, N. Grant, B. and Weste, G. (1989) – Cellular and histological changes induced by *Phytophthora cinnamomi* in a group of plant species ranging from fully susceptible to fully resistant. *Phytopathology* **79** (4): 417-424.

Cahill, D.M., Bennett, I.J. and McComb, J.A. (1992) – Resistance of micropropagated *Eucalyptus marginata* to *Phytophthora cinnamomi*. *Plant Disease* **76** (6): 630-632.

Cahill, D.M. and McComb, J.A. (1992) – A comparison of changes in phenylalanine ammonia-lyase activity, lignin and phenolic synthesis in the roots of *Eucalyptus calophylla* (field resistant) and *E. marginata* (susceptible) when infected with *Phytophthora cinnamomi*. *Physiological and Molecular Plant Pathology* **40**: 315-332.

Cahill, D.M., Bennett, I.J. and McComb, J.A. (1993) – Mechanisms of resistance to *Phytophthora cinnamomi* in clonal, micropropagated *Eucalyptus marginata*. *Plant Pathology* **42**: 865-872.

Cahill, D.M. and Hardham, A.R. (1994) – A dipstick immunoassay for the specific detection of *Phytophthora cinnamomi* in soils. *Phytopathology* **84**: 1284-1292.

Cahill, D. (1995) – Review of *Phytophthora* Diseases in Australia. A report for Rural Industries Research and Development Corporation. Research Paper 93/4.

Cahill, D.M. (1999) – Detection, identification and disease diagnosis of soilborne pathogens. *Australasian Plant Pathology* 28: 34-44.

Candela, M.E., Alcazar, M.D., Espin, A., Egea, C. and Almela, L. (1995) – Soluble phenolic acids in *Capsicum annuum* stems infected with *Phytophthora capsici*. *Plant Pathology* **44**: 116-123.

Carbon, B.A., Bartle, G.A. and Murray, A.M. (1981) – Water stress, transpiration and leaf area index in eucalypt plantations in a bauxite mining area in south-west Australia. *Australian Journal of Ecology* **6**: 459-466.

Carlile, M.J. (1983) – Motility, Taxis, and Tropism in *Phytophthora*. In 'Phytophthora – Its Biology, Taxonomy, Ecology and Pathology' Eds. D.C. Erwin. S Bartnicki-Garcia and P.H. Tsao. The American Phytopathological Society, St Paul, Minnesota USA. pp 95-107.

Carrasco, A., Boudet, A.M. and Marigo, G. (1978) – Enhanced resistance of tomato plants to *Fusarium* by controlled stimulation of their natural phenolic production. *Physiological Plant Pathology* **12**: 225-232.

Casares, A., Melo, E.M.P.F., Ferraz, J.F.P. and Ricardo, C.P.P. (1986) – Differences in ability of *Phytophthora cambivora* and *P. cinnamomi* to dephenolize lignin. *Transactions of the British Mycological Society* **87**: 229-235.

Chakraborty, S., Murray, G.M., Magarey, P.A., Yonow, T., O'Brien, R.G., Croft, B.J., Barbetti, M.J., Sivasithamparam, K., Old, K.M., Dudzinski, M.J., Sutherst, R.W., Penrose, L.J., Archer, C. and Emmett, R.W. (1998) – Potential impact of climate change on plant diseases of economic significance to Australia. *Australasian Plant Pathology* **27**: 15-35.

Chee, K.H. and Newhook, F.J. (1965) – Nutritional studies with *Phytophthora cinnamomi* Rands. *New Zealand Journal of Agricultural Research* **8**: 523-529.

Chee, K.H. and Newhook, F.J. (1966) – Relationship of microorganisms to sporulation of *Phytophthora cinnamomi* Rands. *New Zealand Journal of Agricultural Research* **9**: 32-34.

Collins, S., Shearer, B., McComb, J. Colquhoun, I. and Hardy, G. (2001) – Long-term survival of *Phytophthora cinnamomi* in organic matter under different soil moisture conditions. Second International IUFRO Meeting Albany 2001 (Abstract).

Colquhoun, I.J., Ridge, R.W., Bell, D.T., Loneragan, W.A. and Kuo, J. (1984) – Comparative studies in selected species of *Eucalyptus* used in rehabilitation of the northern jarrah forest Western Australia. I. Patterns of xylem pressure potential and diffusive resistance of leaves. *Australian Journal of Botany* **32**: 367-373.

Colquhoun, I.J. and Petersen, A.E. (1994) – The impact of plant disease on mining. *Journal of the Royal Society of Western Australia* **77**: 151-158.

Colquhoun, I.J. and Hardy, G.E.St. J. (2000) – Managing the risks of Phytophthora Root and Collar Rot during Bauxite Mining in the *Eucalyptus marginata* (Jarrah) Forest of Western Australia. *Plant Disease* **82** (2): 116-127.

Crombie, D.S., and Milburn, J.A. (1988) – Water relations of rural eucalypt dieback. *Australian Journal of Botany* **36**: 233-237.

Crombie, D.S. and Tippett, J.T. (1990) – A comparison of water relations, visual symptoms, and changes in stem girth for evaluating impact of *Phytophthora cinnamomi* dieback on *Eucalyptus marginata*. *Canadian Journal of Forest Research* **20**: 233-240.

Crombie, D.S. and Bunny, F.J. (1994) – Disease and forest production in Western Australia with particular reference to the effects of *Phytophthora cinnamomi*. *Journal of the Royal Society of Western Australia* **77**: 145-149.

Cruickshank, I.A.M. (1963) – Phytoalexins. *Annual Review of Phytopathology* **1**: 351-374.

Davison, E.M. and Tay, F.C.S. (1985) – The effect of waterlogging on seedlings of *Eucalyptus marginata*. *New Phytologist*, **101**: 743-753.

Davison, E.M. and Tay, F.C.S. (1988) – Annual increment of *Eucalyptus marginata* trees on sites infested with *Phytophthora cinnamomi*. *Australian Journal of Botany* 36: 101-106.

Davison, E.M. (1994) – Role of environment in dieback of jarrah: Effects of waterlogging on jarrah and *Phytophthora cinnamomi*, and infection of jarrah by *P. cinnamomi*. *Journal of the Royal Society of Western Australia* **77**: 123-126.

Davison, E.M., Stukely, M.J.C., Crane, C.E. and Tay, F.C.S. (1994) – Invasion of Phloem and Xylem of Woody Stems and Roots of *Eucalyptus marginata* and *Pinus radiata* by *Phytophthora cinnamomi*. *Phytopathology* **84** (4): 335-430.

Davison, E.M. (1997) – Are jarrah (*Eucalyptus marginata*) trees killed by *Phytophthora cinnamomi* or waterlogging? *Australian Forestry* **60** (2): 116-124.

Dawson, P. and Weste, G. (1984) – Impact of root infection by *Phytophthora cinnamomi* on the water relations of two *Eucalyptus* species that differ in susceptbility. *Phytopathology* **74**: 486-490.

Dell, B. Bartle, J.R. and Tacey, W.H. (1983) – Root occupation and root channels of jarrah forest subsoils. *Australian Journal of Botany* **31**: 615-627.

Dell, B., Havel, J.J. and Malajczuk, N. (1989) - The Jarrah Forest. A complex mediterranean ecosystem. Dordrecht: Kluwer Academic Publishers.

Dell, B. and Malajczuk, N. (1989) – Jarrah dieback – A disease caused by *Phytophthora cinnamomi*. In: 'The Jarrah Forest. A complex mediterranean ecosystem' (Eds. B. Dell, J.J. Havel and N. Malajczuk.) pp 67-87. Dordrecht: Kluwer Academic Publishers.

Dimond, A.E. (1970) – Biophysics and biochemistry of the vascular wilt syndrome. *Annual Review of Phytopathology* **8**: 301-322.

Dixon, R.A., Harrison, M.J. and Lamb, C.J.(1994) – Early events in the activation of plant defence responses. *Annual Review of Phytopathology* **32**: 479-501.

Dobrowolski, M.P. and O'Brien, P.A. (1993) – Use of RAPD-PCR to isolate a species-specific DNA probe for *Phytophthora cinnamomi*. *FEMS Microbiology Letters* **113**: 43-48.

Dobrowolski, M.P. (1999) – Population and sexual genetics of *Phytophthora cinnamomi* in Australia using microsatellite markers. PhD Thesis, Murdoch University, Perth, Western Australia.

Dobrowolski, M.P., Tommerup, I.C., O'Brien, P.A., Shearer, B.L., Colquhoun, I.J. and Hardy, G.E.St.J. (2003) – The potential of *Phytophthora cinnamomi* to develop resistance to chemical control by phosphite – 8th International Congress of Plant Pathology, Christchurch, New Zealand. February, 2003 (Poster).

Dolan, T.E. and Coffey, M.D. (1986) – Laboratory screening technique for assessing resistance of four avocado rootstocks to *Phytophthora cinnamomi*. *Plant Disease* **70**: 115-118.

Doley, D. (1967) – Water relations of *Eucalyptus marginata* Sm. under natural conditions. *Ecology* **55**: 597-614.

D'Souza, N.K. (2001) – Biological control of *Phytophthora cinnamomi*: the potential of Western Australian native legumes to protect susceptible plant species. Honours Thesis, Murdoch University, Perth, Western Australia.

Dudzinski, M.J., Old, K.M. and Gibbs, R.J. (1993) – Pathogenic variability in Australian isolates of *Phytophthora cinnamomi*. *Australian Journal of Botany* **41**: 721-732.

Duncan, M.J. and Keane, P.J. (1996) – Vegetation changes associated with *Phytophthora cinnamomi* and its decline under *Xanthorrhoea australis* in Kinglake National Park, Victoria. *Australian Journal of Botany* **44**, 355-369.

Duniway, J.M. (1976) – Water status and imbalance. In 'Encyclopedia of Plant Physiology' Vol. IV. (Editors: A. Pirson and M.H. Zimmermann.) Springer-Verlag, New York. pp 430-449.

Duniway, J.M. (1979) – Water relations of water molds. *Annual Review of Phytopathology* **17**: 431-460.

Duniway, J.M. (1983) – Role of physical factors in the development of *Phytophthora* diseases. In Phytophthora – Its Biology, Taxonomy, Ecology and Pathology' Eds. D.C. Erwin. S Bartnicki-Garcia and P.H. Tsao. The American Phytopathological Society, St Paul, Minnesota USA. pp 175- 187.

Erwin, D.C., Bartnicki-Garcia, S. and Tsao, P.H. (Eds.) (1983) 'Phytophthora – Its Biology, Taxonomy, Ecology and Pathology.' The American Phytopathological Society, St Paul, Minnesota USA.

Erwin, DC. (1995) Variability Within and Among Species of *Phytophthora*. In 'Phytophthora – Its Biology, Taxonomy, Ecology and Pathology' Eds. D.C. Erwin. S Bartnicki-Garcia and P.H. Tsao. The American Phytopathological Society, St Paul, Minnesota USA.

Erwin, D.C. and Ribeiro, O.K. (1996) – Phytophthora Diseases Worldwide. APS Press. The American Phytopathological Society, Minnesota, U.S.A.

Fan, S. and Blake, T.J. (1997) – Comparison of polyethylene glycol 3350 induced osmotic stress and soil drying for drought simulation in three woody species. *Trees* 11: 342-348.

Gabor, B.K., O'Gara, E.T., Philip, B.A. Horan, D.P. and Hardham, A.R. (1993) – Specificities of monoclonal antibodies to *Phytophthora cinnamomi* in two rapid diagnostic assays. *Plant Disease* **77**: 1189-1197.

Glauert, A.M. (1975) – Fixation, Dehydration and Embedding of Biological Specimens. North-Holland Publishing Co.

Grieve, B.J. (1956) – Studies in the water relations of plants. I. Transpiration of Western Australian (Swan Plain) sclerophylls. *Proceedings of the Royal Society of Western Australia* **40**: 15-30.

Groves, E. (2000) – Role of soluble phenolic compounds from infected *Eucalyptus marginata* in reducing the recovery of *Phytophthora cinnamomi*. Unpublished report for Neville Stanley Studentship at Murdoch University, Perth, Western Australia.

Hardham, A.R., Gubler, F., Duniec, J. and Elliot, J. (1991) – A review of methods for the production and use of monoclonal antibodies to study zoosporic plant pathogens. *Journal of Microscopy* **162**: 305-318.

Hardham, A.R., Cahill, D.M., Cope, M., Gabor, B.K., Gubler, F. and Hyde, G.J. (1994) – Cell surface antigens of *Phytophthora* spores: biological and taxonomic characterization. *Protoplasma* **181**: 213-232.

Hardham, A.R. (2001) – The cell biology behind *Phytophthora* pathogenicity. *Australasian Plant Pathology* **30**: 91-98.

Hardy, G.E.St.J., Colquhoun, I.J. and Nielsen, P. (1996) – The early development of disease caused by *Phytophthora cinnamomi* in *Eucalyptus marginata* and *Eucalyptus calophylla* growing in rehabilitated bauxite mined areas. *Plant Pathology* **45**: 944-954.

Hardy, G.E. St J., Barrett, S. and Shearer, B.L. (2001) – The future of phosphite as a fungicide to control the soilborne plant pathogen *Phytophthora cinnamomi* in natural ecosystems. *Australasian Plant Pathology* **30** (2): 133-139.

Havel, J.J. (1975a) – Site-vegetation mapping in the northern jarrah forest (Darling range). I. Definition of site-vegetation types. Bulletin 86, Forests Department, Western Australia (cited by Shearer and Tippett, 1989).

Havel, J.J. (1975b) – Site-vegetation mapping in the northern jarrah forest (Darling range). 2. Location and mapping of site-vegetation types, Bulletin 87, Forests Department, Western Australia (cited by Shearer and Tippett, 1989).

Havel, J.J. (1989) - Land use conflicts and the emergence of multiple land use. In: *The Jarrah Forest. A complex mediterranean ecosystem*. (Eds. B. Dell, J.J. Havel and N. Malajczuk.) pp 67-87. Dordrecht: Kluwer Academic Publishers.

Hickman, C.J. (1970) – Biology of *Phytophthora* zoospores. *Phytopathology* **60**: 1128-1135.

Hill, R.S. (1994) – The history of selected Australian taxa. In 'History of the Australian vegetation: Cretaceous to Recent.' (Ed. R.S. Hill.) Cambridge University Press, Cambridge, U.K. pp. 390-415.

Hoagland, D.R. and Arnon, D.I. (1950) – The water-culture method for growing plants without soil. California Agricultural Experimental Station Circular 347. http://ic.ucsc.edu (also cited by Kannewischer and Mitchell, 1981).

Hüberli, D. (1995) – Analysis of variability among isolates of *Phytophthora cinnamomi* Rands from *Eucalyptus marginata* Donn ex. Sm and *E. calophylla* R. Br. based on Cultural Characteristics, Sporangia and Gametangia Morphology and Pathogenicity. Honours Thesis, Murdoch University, Perth, Western Australia.

Hüberli, D., Hardy, G.E.St.J., Tommerup, I.C. and Colquhoun, I.J.(1997) Temperature changes resistance of clonal *Eucalyptus marginata* to *Phytophthora cinnamomi*. In Eleventh Biennial Conference of the Australasian Plant Pathology Society: 137. Perth, Western Australia.

Hüberli, D., Tommerup, I.C. and Hardy, G.E.St.J. (2000) – False-negative isolations or absence of lesions may cause misdiagnosis of diseased plants infected with *Phytophthora cinnamomi*. *Australasian Plant Pathology* **29**: 164-169.

Hüberli, D., Tommerup, I.C., Calver, M.C., Colquhoun, I.J. and Hardy, G.E.St.J. (2002) – Temperature and inoculation method influence disease phenotypes and mortality of *Eucalyptus marginata* clonal lines inoculated with *Phytophthora cinnamomi*. *Australasian Plant Pathology* **31**: 107-118.

Hüberli, D., Tommerup, I.C., Dobrowolski, M.P., Calver, M.C. and Hardy, G.E.St.J. (2001) – Phenotypic variation in a clonal lineage of two *Phytophthora cinnamomi* populations from Western Australia. *Mycological Research* **105** (9): 1053-1064.

Irwin, J.A.G., Cahill, D.M. and Drenth, A. (1995) – *Phytophthora* in Australia. *Australian Journal of Agricultural Research* **46**: 1311-1337.

Jeffers, S.N. and Martin, S.B. (1986) – Comparison of two media selective for *Phytophthora* and *Pythium* species. *Plant Disease* **70**: 1038-1043.

Jeffrey, S.I.B., Jinks, J.L. and Grindle, M. (1962) – Intraracial variation in *Phytophthora infestans* and field resistance to potato blight. *Genetica* **32**: 323-338.

Jensen, W.A. (1962) – Botanical Histochemistry: Principles and Practice. W.H. Freeman and Co. San Francisco, USA (cited by O'Brien, T.P and McCully, M.E.

(1981) in 'The Study of Plant Structure: Principles and Selected Methods.' Termarcarphi Pty. Ltd., Melbourne Australia.

Kannewischer, M.E. and Mitchell, D.J. (1981) – Relationships of numbers of spores of *Phytophthora parasitica* var. *nicotianae* to infection and mortality of tobacco. *Phytopathology* **71**: 69-73.

Karnovsky, M.J. (1965) – A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. *Journal of Cell Biology* **27**: 137A-138A.

Kinal, J., Shearer, B.L. and Fairman, R.G. (1993) – Dispersal of *Phytophthora cinnamomi* through lateritic soil by laterally flowing subsurface water. *Plant Disease* **77** (11): 1085-1090.

Koch, J.M. and Ward, S.C. (1994) – Establishment of understorey vegetation for rehabilitation of bauxite-mined areas in the jarrah forest of Western Australia. *Journal of Environmental Management* **41**: 1-15.

Kosuge, T. (1969) – The role of phenolics in host response to infection. *Annual Review of Phytopathology* **7**: 195-222.

Ladiges, P.Y. (1997) – Phylogenetic history and classification of eucalypts. In 'Eucalypt Ecology – Individuals to ecosystems.' (Eds. J.E. Williams and J.C. Woinarski) Cambridge University Press, Cambridge, U.K.

Landsberg, J. and Wylie, F.R. (1983) – Water stress, leaf nutrients and defoliation: a model of dieback of rural eucalypts. *Australian Journal of Ecology* **8**: 27-41.

Leach, S.S. and Rich, A.E. (1969) – The possible role of parasexuality and cytoplasmic variation in race differentiation in *Phytophthora infestans*. *Phytopathology* **59**: 1360-1365.

Linde, C., Drenth, A. and Wingfield, M.J. (1999) – Gene and genotypic diversity of *Phytophthora cinnamomi* in South Africa and Australia revealed by DNA polymorphisms. *European Journal of Plant Pathology* **105**: 667-680.

Lowry, O.H. (1951) - Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry* **193**: 265-268.

Lucas, A. Colquhoun, I.J., McComb, J.A. and Hardy, G.E. St.J. (2002) – A new, rapid and non-invasive technique to inoculate plants with *Phytophthora cinnamomi*. *Australasian Plant Pathology* **31** (1): 27-30.

Luque, J., Cohen, M., Save, R., Biel, C. and Alvarez, I. (1999) – Effects of three fungal pathogens on water relations, chlorophyll fluorescence and growth of *Quercus suber* L. *Ann. For. Sci*. **56**: 19-26.

Malajczuk, N. (1983) – Microbial antagonism to *Phytophthora*. In: '*Phytophthora* – Its Biology, Taxonomy, Ecology and Pathology' Eds. DC Erwin. S Bartnicki-Garcia and

PH Tsao. The American Phytopathological Society, St Paul, Minnesota USA. pp 197-218.

Marcais, B., Dupuis, F. and Desprezloustau, M.L. (1993) – Influence of water stress on susceptibility of red oak (*Quercus rubra*) to *Phytophthora cinnamomi*. *European Journal of Forest Pathology* **23** (5): 295-305.

Marks, G.C., Kassaby, F.Y. and Fagg, P.C. (1975) – Variation in population levels of *Phytophthora cinnamomi* in *Eucalyptus* forest soils of eastern Victoria. *Australian Journal of Botany* **23**: 435-439.

Marks, G.C., Smith, I.W. and Kassaby, F.Y. (1981) – Trunk Infection of *Eucalyptus* species by *Phytophthora cinnamomi* Rands: A Preliminary Report. *Australian Forest Research* **11**: 257-267.

Masago, H., Yoshikawa, M., Fukada, M. and Nakanishi, N. (1977) – Selective inhibition of *Pythium* spp. on a medium for direct isolation of *Phytophthora* spp. from soils and plants. *Phytopathology* **67**: 425-428.

McChesney, C.J., Koch, J.M. and Bell, D.T. (1995) – Jarrah Forest Restoration in Western Australia: Canopy and Topographic effects. *Restoration Ecology* **3** (2): 105-110.

McComb, J.A., Bennett, I.J., Stukely, M.J.C. and Crane, C. (1990) – Selection and propagation of jarrah for dieback resistance - a progress report. *Combined proceedings of the International Plant Propagators' Society* **40**: 86-90.

McComb, J.A., Stukely, M. and Bennett, I.J. (1994) – Future ecosystems – use of genetic resistance. *Journal of the Royal Society of Western Australia* **77**: 179-180.

McDougall, K.L., Hardy, G.E.St.J. and Hobbs, R.J. (2001) – Additions to the host range of *Phytophthora cinnamomi* in the jarrah (*Eucalyptus marginata*) forest of Western Australia. *Australian Journal of Botany* **49**: 193-198.

McDougall, K.L., Hardy, G.E.St.J. and Hobbs, R.J. (2002) – Distribution of *Phytophthora cinnamomi* in the northern jarrah (*Eucalyptus marginata*) forest of Western Australia in relation to dieback age and topography. *Australian Journal of Botany* **50**: 107-114.

Mehrlich, F.P. (1935) – Nonsterile soil leachate stimulating zoosporangia production by *Phytophthora* sp. *Phytopathology* **25**: 432-435.

Moerschbacher, B.M., Noll, U.M. Flot, B.E. and Reisner, H.J. (1988) – Lignin biosynthetic enzymes in stem rust infected, resistant and susceptible near-isogenic wheat lines. *Physiological and Molecular Plant Pathology* **33**: 33-46..

Mohammed, G.H., Binder, W.D. and Gillies, S.L. (1995) – Chlorophyll fluorescence: A review of its practical forestry applications and instrumentation. *Scandanavian Journal of Forest Research* **10**: 383-410.

Morgan, J.M. (1984) – Osmoregulation and water stress in higher plants. *Annual Review of Phytopathology* **35**: 299-319.

Mulcahy, M.J., Churchward, H.M. and Dimock, G.M. (1972) – Land form and soils on an uplifted peneplain in the Darling range, Western Australia. *Australian Journal of Soil Research* **10**: 1-14.

Myers, B.A. and Neales, T.F. (1986) – Osmotic adjustment, induced by drought, in seedlings of three *Eucalyptus* species. *Australian Journal of Plant Physiology* **13**: 597-603.

Nemchin, A.A. and Pidgeon, R.T. (1999) – U-Pb ages on titanite and apatite from the Darling Range granite: implications for Late Archaean history of the southwestern Yilgarn Craton. *Precambrian Research* 96: 125-139.

Nesbitt, H.J., Malajczuk, N. and Glenn, A.R. (1979a) – Effect of organic matter on the survival of *Phytophthora cinnamomi* Rands in soil. *Soil Biology and Biochemistry* **11**: 133-136.

Nesbitt, H.J., Malajczuk, N. and Glenn, A.R. (1979b) – Effect of soil moisture and temperature on the survival of *Phytophthora cinnamomi* Rands in soil. *Soil Biology and Biochemistry* **11**: 137-140.

Newhook, F.J. and Podger, F.D. (1972) – The role of *Phytophthora cinnamomi* in Australian and New Zealand forests. *Annual Review of Phytopathology* **10**: 299-326.

Nichols, O.G., Carbon, B.A., Colquhoun, I.J., Croton, J.T. and Murray, N.J. (1985) – Rehabilitation after bauxite mining in south-western Australia. Landscape Planning 12: 75-92.

Nicholson, R.L. and Hammerschmidt, R. (1992) – Phenolic compounds and their role in disease resistance. *Annual Review of Phytopathology* **30**: 369-389.

O'Gara, E., Hardy, G.E.St.J., and McComb, J.A (1996) – The ability of *Phytophthora cinnamomi* to infect through unwounded and wounded periderm tissue of *Eucalyptus marginata*. *Plant Pathology* **45**: 955-963.

O'Gara, E., Mc Comb, J.A. Colquhoun, I.J. and Hardy, G.E.St.J. (1997) – The infection of non-wounded and wounded periderm tissue at the lower stem of *Eucalyptus marginata* by zoospores of *Phytophthora cinnamomi*, in a rehabilitated bauxite mine. *Australasian Plant Pathology* **26**: 135-141.

O'Gara, E. (1998) – Infection and disease of *Eucalyptus marginata* (Jarrah), caused by *Phytophthora cinnamomi* in rehabilitated bauxite mines in the south-west of Western Australia. PhD Thesis, Murdoch University, Perth, Western Australia.

Okey, E.N., Duncan, E.J., Sirju-Charran, G. and Sreenivasan, T.N. (1997) – *Phytophthora* canker resistance in cacao; role of peroxidase, polyphenoloxidase and phenylalanine ammonia-lyase. *Journal of Phytopathology* **145**: 295-299.

Old, K.M. (Ed.) (1978) - Phytophthora and Forest Management in Australia. Report of a Conference held at CSIRO Division of Forest Research Canberra 18-20 October, 1978.

Old, K.M., Oros, J.M. and Malafant, K.W. (1984) – Survival of *Phytophthora cinnamomi* in root fragments in Australian forest soils. *Transactions of the British Mycological Society* **82**: 605-613.

Old, K.M., Dudzinski, M.J. and Bell, J.C. (1988) – Isozyme variability in field populations of *Phytophthora cinnamomi* in Australia. *Australian Journal of Botany* **36**: 355-360.

Pate, J. and Arthur, D. (1998) – $\delta^{13}C$ analysis of phloem sap carbon: novel means of evaluating seasonal water stress and interpreting carbon isotope signatures of foliage and trunk wood of *Eucalyptus globulus*. *Oecologia* **117**: 301-311.

Pate, J., Shedley, E., Arthur, D. and Adams, M. (1998) – Spatial and temporal variations in phloem sap composition of plantation-grown *Eucalyptus globulus*. *Oecologia* **117**: 312-322.

Pearce, R.B. (1996) – Antimicrobial defences in the wood of living trees. *New Phytologist* **132**: 203-233.

Phillips, D. and Weste, G. (1985) – Growth rates of four Australian isolates of *Phytophthora cinnamomi* in relation to temperature. *Transactions of the British Mycological Society* **84**: 183-185.

Pilbeam, R.A., Colquhoun, I.J., Shearer, B. and Hardy, G.E. St J. (2000) – Phosphite concentration; its effect on phytotoxicity symptoms and colonisation by *Phytophthora cinnamomi* in three understorey species of *Eucalyptus marginata* forest. *Australasian Plant Pathology* **29**: 86-95.

Podger, F.D., Doepel, R.F. and Zentmyer, G.A. (1965) – Association *of Phytophthora cinnamomi* with a disease of *Eucalyptus marginata* forest in Western Australia. *Plant Disease Reporter* **49**: 943-947.

Podger, F.D. (1968) - Aetiology of jarrah dieback - a disease of dry sclerophyll *Eucalyptus marginata* Sm. forests in Western Australia. MSc Thesis, University of Melbourne, Melbourne, Australia.

Podger, F.D. and Batini, F. (1971) – Susceptibility to *Phytophthora cinnamomi* root-rot of thirty six species of *Eucalyptus*. *Australian Forest Research* **5** (3): 9-20.

Podger, F.D. (1972) – *Phytophthora cinnamomi*, a cause of lethal disease in indigenous plant communities in Western Australia. *Phytopathology* **62**: 972-981.

Pratt, B.H. and Heather, W.A. (1973) – The origin and distribution of *Phytophthora cinnamomi* Rands in Australian native plant communities and the significance of its

association with particular plant species. *Australian Journal of Biological Science* **26**: 559-573.

Rands, R.D. (1922) – Streepkanker van kaneel, veroorzaakt door *Phytophthora cinnamomi* n. sp. (Stripe canker of cinnamon caused by *Phytophthora cinnamomi*) Mededeelingen van het Instituut voor Plantenziekten. Department van Landbouw, Nijverheid en Handel 54: 41. (cited by Zentmyer, 1980).

Reeves, R.J. (1975) – Behaviour of *Phytophthora cinnamomi* Rands in different soils and water regimes. *Soil Biology and Biochemistry* **7**: 19-24.

Retig, N. (1974) - Changes in peroxidase and polyphenoloxidase associated with natural and induced resistance of tomato to *Fusarium* wilt. *Physiological Plant Pathology* **4**: 145-150.

Retig, N. and Chet, I. (1974) – Catechol-induced resistance of tomato plants to *Fusarium* wilt. *Physiological Plant Pathology* **4**: 469-475.

Ritchie, G.A. and Hinckley, T.M. (1975) – The pressure chamber as an instrument for ecological research. *Advances in Ecological Research* **9**: 165-254.

Robin, C., Capron, G. and Desprez-Loustau, M.L. (2001) - Root infection by *Phytophthora cinnamomi* in seedlings of three oak species, *Plant Pathology* **50**: 708-716.

Roche, S., Koch, J.M. and Dixon, K.W. (1997) – Smoke-enhanced seed germination for mine rehabilitation in the southwest of Western Australia. *Restoration Ecology* **5** (3): 191-203.

Rokich, D.P. and Bell, D.T. (1995) – Light Quality and Intensity Effects on the Germination of Species from the Jarrah (*Eucalyptus marginata)* Forest of Western Australia. *Australian Journal of Botany* **43**: 169-179.

Rosenthal, G.A. (1986) – The Chemical Defenses of Higher Plants. *Scientific American* **254**: 76-81.

Scheiner, S.M. and Rey-Benayas, J.M. (1994) – Global patterns of plant diversity. *Evolutionary Ecology* **8**: 331-347.

Schmitthenner, A.F. and Canaday, C.H. (1983) – Role of Chemical Factors in Development of *Phytophthora* Diseases. In '*Phytophthora* – Its Biology, Taxonomy, Ecology and Pathology' Eds. D.C. Erwin. S Bartnicki-Garcia and P.H. Tsao. The American Phytopathological Society, St Paul, Minnesota USA.

Schoeneweiss, D.F. (1975) – Predisposition, stress and plant disease. *Annual Review of Phytopathology* **13**: 193-211.

Scholander, P.F., Hammel, H.T., Bradstreet, E.D. and Hemmingsen, E.A. (1965) – Sap pressure in vascular plants. *Science* **148**: 339-346.

Schur, B. (1985) – Jarrah forest or bauxite dollars? A critique of bauxite mining rehabilitation in the jarrah forests of southwestern Australia. Save Native Forests (W.A.), Perth, Western Australia.

Shea, S.R. (1979) – *Phytophthora cinnamomi* Rands – a collar rot pathogen of *Banksia grandis* Willd. *Australasian Plant Pathology* **8**: 32-34.

Shea, S.R., Shearer, B.L., Tippett, J.T. and Deegan, P.M. (1983) – Distribution, reproduction and movement of *Phytophthora cinnamomi* on sites highly conducive to jarrah dieback in south Western Australia. *Plant Disease* **67**: 970-973.

Shearer, B.L. and Shea, S.R. (1987) – Variation in Seasonal Population Fluctuations of *Phytophthora cinnamomi* within and between Infected *Eucalyptus marginata* Sites of Southwestern Australia. *Forest Ecology and Management* **21**: 209-230.

Shearer, B.L., Michaelsen, B.J. and Warren, H.J. (1987) – Comparative Behaviour of *Phytophthora* Species in the Secondary Phloem of Stems and Excised Roots of *Banksia grandis* and *Eucalyptus marginata*. *Australian Journal of Botany* **35**: 103-110.

Shearer, B.L. and Tippett, J.T. (1989) – Jarrah Dieback: The Dynamics and Management of *Phytophthora cinnamomi* in the Jarrah (*Eucalyptus marginata*) Forest of South-western Australia. Research Bulletin No.3, Department of Conservation and Land Management, Western Australia.

Shearer, B.L. and Dillon, M. (1995) - Susceptibility of plant species in *Eucalyptus marginata* forest to infection by *Phytophthora cinnamomi.* *Australian Journal of Botany* **43**: 113-134

Shearer, B.L. and Fairman, R.G. (1997) – Phosphite inhibits lesion development of *Phytophthora cinnamomi* for at least four years following trunk injection of *Banksia* species and *Eucalyptus marginata*. In Proceedings of the 11th biennial conference of the Australasian Plant Pathology Society, Perth, Western Australia.

Shepherd, C.J. (1975) – *Phytophthora cinnamomi*–an ancient immigrant to Australia. *Search* **6**: 484-490.

Shigo, A. (1984) – Compartmentalization; A conceptual framework for understanding how trees grow and defend themselves. *Annual Review of Phytopathology* **22**: 189-214.

Schoulties, C.L. and Baker, K.F. (1974) – Improving reproducibility in studies on zoosporangial formation by *Phytophthora cinnamomi*. *Proceedings of the American Phytopathological Society* **1**:60 (cited by Zentmyer, G.A. 1980).

Smith, J.W. and Marks, G.C. (1986) – Effect of Moisture Stress in *Eucalyptus sieberi* on Growth of Lesions caused by *Phytophthora cinnamomi*. *Australian Forest Research* **16**: 273-279.

Soliday, C.L., Dean, B.B. and Kolattukudy, P.E. (1978) – Suberization: inhibition by washing and stimulation by abscisic acid in potato disks and tissue culture. *Plant Physiology* **61**: 170-174.

Specht, R.L. (1969) – Comparison of sclerophyllous vegetation characteristics of mediterranean type climates in France, California and southern Australia. I. Structure, morphology and succesion. *Australian Journal of Botany* **17**: 277-292.

Stoneman, G. (1992) – Factors affecting the establishment of jarrah (*Eucalyptus marginata)* from seed in the northern jarrah forest of Western Australia. PhD Thesis, Murdoch University, Perth, Western Australia.

Stoneman, G.L., Turner, N.C. and Dell, B. (1994) – Leaf growth, photosynthesis and tissue water relations of greenhouse-grown *Eucalyptus marginata* seedlings in response to water deficits. *Tree Physiology* **14**: 633-646.

Stukely, M.J.C. and Crane, C.E. (1994) – Genetically based resistance of *Eucalyptus marginata* to *Phytophthora cinnamomi*. *Phytopathology* **84** (6): 650-656.

Tippett, J.T. and Hill, T.C. (1983) – The relationship between bark moisture and invasion of *Eucalyptus marginata* by *Phytophthora cinnamomi*. *Australasian Plant Pathology* **12**: 40-41.

Tippett, J.T., Shea, S.R., Hill, T.C. and Shearer, B.L. (1983) – Development of Lesions caused by *Phytophthora cinnamomi* in the Secondary Phloem of *Eucalyptus marginata*. *Australian Journal of Botany* **31**: 197-210.

Tippett, J.T., Hill, T.C. and Shearer, B.L. (1985) – Resistance of *Eucalyptus* spp. to invasion by *Phytophthora cinnamomi*. *Australian Journal of Botany* **33**; 409-418.

Tippett, J.T., Crombie, D.S. and Hill, T.C. (1987) – The effect of phloem water relations on the growth of *Phytophthora cinnamomi* in *Eucalyptus marginata*. *Phytopathology* **77**: 246-250.

Tsao, P.H. and Ocana, G. (1969) – Selective isolation of species of *Phytophthora* from natural soils on an improved antibiotic medium. *Nature* **223**: 636-638.

Tsao, P.H. and Guy, S.O. (1977) - Inhibition of *Mortierella* and *Pythium* in a *Phytophthora*-isolation medium containing hymexazol. *Phytopathology* **67**: 796-801.

Turner, N.C. and Jones, M.M. (1980) – Turgor maintenance by osmotic adjustment: A review and evaluation. In 'Adaptation of plants to water and high temperature stress.' (Editors: N.C. Turner and P.J. Kramer.) John Wiley and Sons Ltd: New York. pp 87-103.

Turner, N.C. (1981) – Techniques and experimental approaches for the measurement of plant water status. *Plant and Soil* **58**: 339-366.

Tynan, K.M., Wilkinson, C.J., Holmes, J.M., Dell, B., Colquhoun, I.J., McComb, J.A. and Hardy, G.E. St J. (2001) – The long-term ability of phosphite to control

Phytophthora cinnamomi in two native plant communities of Western Australia. *Australian Journal of Botany* **49**: 761-770.

van West, P., Morris, B.M., Reid, B., Appiah, A.A., Osborne, M.C. Campbell, T.A. Shepherd, S.J. and Gow, N.A.R. (2002) – Oomycete plant pathogens use electric fields to target roots. *Molecular Plant-Microbe Interactions* **15** (8): 790-798.

Vance, C.P., Kirk, T.K. and Sherwood, R.T. (1980) – Lignification as a mechanism of disease resistance. *Annual Review of Phytopathology* **18**: 259-288.

Vidhyasekaran, P. (1997) – Fungal pathogenesis in plants and crops: molecular biology and host defence mechanisms. Marcel Dekker: New York, N.Y. 10016.

Ward, S.C., Slessar, G.C. and Glenister, D.J. (1993) – Environmental resource management practices of Alcoa of Australia Limited. In "Australasian Mining and Metallurgy" (Eds. J.T. Woodcock and J.K. Hamilton) University of NSW Press: Sydney. pp104-108.

Ward, S.C. and Koch, J.M. (1996) – Biomass and nutrient distribution in a 15.5 year old forest growing on a rehabilitated bauxite mine. *Australian Journal of Ecology* **21**: 309-315.

Ward, S.C., Koch, J.M. and Ainsworth, G.L. (1996) – The effect of timing on rehabilitation procedures on the establishment of a jarrah forest after bauxite mining. *Restoration Ecology* **4** (10): 19-24.

Wardell-Johnson, G.W., Williams, J.E., Hill, K.D. and Cumming, R. (1997) – Evolutionary biogeography and contemporary distribution of eucalypts. In 'Eucalypt Ecology - Individuals to ecosystems.' (Eds. J.E. Williams and J.C. Woinarski) Cambridge University Press, Cambridge, U.K.

Wargo, P.M. (1996) - Consequences of environmental stress on oak: predisposition to pathogens. *Ann. Sci. For*. **53**: 359-368 (cited by Luque *et al*., 1999).

Weste, G. and Vithanage, K. (1979) – Survival of chlamydospores of *Phytophthora cinnamomi* in several non-sterile, host-free forest soils and gravels at different soil water potentials. *Australian Journal of Botany* **27**: 693-701.

Weste, G. (1983) – Population Dynamics and Survival of *Phytophthora*. In 'Phytophthora – Its Biology, Taxonomy, Ecology and Pathology' Eds. D.C. Erwin. S Bartnicki-Garcia and P.H. Tsao. The American Phytopathological Society, St Paul, Minnesota USA. pp 237-257.

Weste, G. and Marks, G.C. (1987) – The biology of *Phytophthora cinnamomi* in Australasian forests. *Annual Review of Phytopathology* **25**: 207-229.

Weste, G. and Ashton, D.H. (1994) – Regeneration and survival of indigenous dry sclerophyll species in the Brisbane Ranges, Victoria, after a *Phytophthora cinnamomi* epidemic. *Australian Journal of Botany* **42**: 239-383.

Weste, G. and Kennedy, J. (1997) – Regeneration of susceptible native species following a decline of *Phytophthora cinnamomi* over a period of 20 years on defined plots in the Grampians, Western Victoria. *Australian Journal of Botany* **45**: 167-190.

Wilkinson, C.J., Holmes, J.M., Tynan, K.M., Colquhoun, I.J., McComb, J.A., Hardy, G.E.St.J and Dell, B. (2001) – Ability of phosphite applied in a glasshouse trial to control *Phytophthora cinnamomi* in five plant species native to Western Australia. *Australasian Plant Pathology* **30**: 343-351.

Wills, R.T. (1992) – The ecological impact of *Phytophthora cinnamomi* in the Stirling Range National Park, Western Australia. *Australian Journal of Ecology* **17**: 145-159.

Zentmyer, G.A. (1960) – *Phytophthora* Canker of Macadamia Trees in California. *Plant Disease Reporter* **44**: 819.

Zentmyer, G.A. and Mircetich, S.M. (1966) – Saprophytism and persistence in soil by *Phytophthora cinnamomi*. *Phytopathology* **56**: 710-712..

Zentmyer, G.A., Leary, J.V., Klure, L.J. and Grantham, G.L. (1976) – Variability in growth of *Phytophthora cinnamomi* in relation to temperature. *Phytopathology* **66**: 982-986.

Zentmyer, G.A. (1980) - *Phytophthora cinnamomi* and the Diseases It Causes. Monograph No. 10. The American Phytopathological Society.

Zentmyer, G.A. (1988) – Origin and distribution of four species of *Phytophthora*. *Transactions of the British Mycological Society* **91**: 367-378.